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13. ABSTRACT *(Maximum 200 Words)*

This case-control study examines breast cancer risk in relation to lifetime alcohol consumption. Subjects will be 1120 pre-and post-menopausal women, age 35-79, from Erie and Niagara counties in New York State, with incident, pathologically confirmed breast cancer. A total of 2275 controls will be interviewed; controls are randomly selected and frequency matched to cases on age, race and county of residence. Participants complete a computerized interview, which focuses on in-depth lifetime alcohol consumption history. Potential confounding factors are also assessed. A specimen bank is used to store biological samples for future research of serum and urinary markers of hormones, hormone metabolites, vitamins, genetic polymorphisms and blood levels of antioxidants and oxidative stress. This study provides an important opportunity for an efficient examination of alcohol and other risk factors, particularly genetic variability, in relation to breast cancer risk, with potential for clarification of a significant public health problem. Since the inception of the study, 971 breast cancer cases and 2,048 controls have been interviewed and blood samples stored for 795 cases and 1,890 controls. Data collection is still underway, therefore there are no conclusions to report at this time.

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INTRODUCTION

This research is an epidemiologic investigation into the role of lifetime alcohol exposure in breast cancer etiology, research of considerable relevance to the issue of breast cancer prevention, providing insight on the role of a modifiable, and common exposure. The primary purpose of this study is to examine the history of alcohol consumption from adolescence through adulthood as a risk factor for pre- and post-menopausal breast cancer in women. We will also examine the possible role of factors such as genetic factors, estrogen receptor status, histology, and use of estrogen replacement therapy among post-menopausal women in mediating the effect of alcohol on breast cancer risk. In this case-control study, we will interview 1120 women (280 premenopausal and 840 postmenopausal), age 35-79 from Erie and Niagara counties in western New York with incident, pathologically confirmed cases of breast cancer are being interviewed. There will be 2275 controls interviewed as well. Controls are randomly selected, those under age 65 from lists provided by the New York State Department of Motor Vehicles, those age 65 and over from enrollment lists of the Health Care Finance Administration. Controls will be frequency matched to cases on age, race, and county. Blood samples are stored in a biological specimen bank for future research. Previous research in this area has relied on relatively crude measures of alcohol consumption, generally not distinguishing infrequent drinkers of larger amounts from individuals who drink smaller quantities more often, and those who drink with meals from those who do not drink with meals. There is some evidence that age when drinking began may also affect risk; studies have not examined in detail characteristics of early drinking. Further, there is some evidence that women may differ in their metabolic response to alcohol depending on genetic polymorphisms for enzymes involved in alcohol metabolism. We recently published a report based on an earlier case-control study of breast cancer that indicated that there were differences in risk by the genotype of alcohol dehydrogenase 3 for pre- but not postmenopausal women (1). We now have preliminary evidence from this study that confirms that finding. When the interviewing is completed, we will be able to look into that finding in more detail because the new study includes a very detailed lifetime alcohol history. Data collection will be completed in May of 2001. After that time, we will begin to analyze and make presentations on the data. We also have had two new grants funded utilizing and enhancing this data set. We have submitted another as well that is pending.

(5) Body of Report

Task 1: Months 1-3: Obtain Institutional Review Board approval for the study at all area hospitals

This task is completed. Annual progress reports are submitted to each hospital and IRB approval has continued unchanged. We previously obtained approval from the Institutional Review Boards of twelve hospitals in the region. One exception is Sisters' Hospital, a major hospital, that will not allow us to use their patient population in spite of long negotiations and considerable effort on the part of both our group and the hospital Institutional Review Board. However, the practice of breast surgeons who see virtually all of the breast cancer patients at that hospital are cooperating fully with the study and are allowing us to contact patients using their clinic records. Contact with physicians and health care professionals and other breast cancer advocates is ongoing to promote continued cooperation with the study.

Task 2: Months 1-3: Finalization of all arrangements for interviewing: training interviewers, necessary preparations of computer interview, printing of the paper section of the questionnaire, obtaining lists of potential controls from the Department of Motor Vehicles and the Health Care Finance Administration, purchase of all necessary supplies and equipment.

This task is completed. We are proceeding with interviews of breast cancer cases and controls.

Training of interviewers and supervision of interviewers regarding their administration of the interview is ongoing. We now have a group of interviewers who have been carefully trained and who have been working on this study for up to four years. We meet regularly with them to discuss any concerns and to continue to standardize procedures.

Task 3. Months 4-45. During years 3 and 4 we will interview 700 cases, making a total of 960 (approximately 215 premenopausal and 745 postmenopausal women). We will continue interviews of postmenopausal white women in years three and four. In addition, we will interview 150 controls.

Cases:

We have achieved the goal of 960. To date, we have interviewed 240 pre-menopausal and 731 post-menopausal women with breast cancer. We have received a no cost extension on our grant and we will continue with interviewing until May of 2001. We expect that we will have interviewed a total of 1120 breast cancer cases (280 pre- and 840 postmenopausal women). The additional interviews will be important for increased power of the study, particularly for the examination of gene-environment interactions.

We ascertain cases by having nurse-casefinders visit hospitals at frequent intervals, the frequency determined by the patient load in that hospital. They examine pathology department records to obtain names of individuals aged 35-79 with a histologically confirmed diagnosis of primary breast cancer, living in Erie and Niagara counties and with no previous history of cancer. Once a case is found, we mail a form for permission to interview to the physician, requesting that the physician indicate approval. In addition, this letter requests verification from the physician of the diagnosis of primary breast cancer. If there is delay, we contact the physician by phone. Once approval is obtained, we invite the patient to participate. Most cases are interviewed within 2-4 months of diagnosis; none are interviewed more than a year after diagnosis. We secure informed consent from all subjects.

We have compared our case ascertainment with data from the New York State tumor registry. Because there is considerable delay in obtaining registry data, those data are not an appropriate tool for identification of cases. We have used the tumor registry to verify the completeness of our case identification. Based on statistics for 1993-7, the number of cases identified in our study are very comparable to those of the tumor registry, exceeding those statistics slightly.

Age and racial distribution of the cases interviewed for this study are provided in detail in Table 1.

Table 1. Characteristics of Breast Cancer Cases Interviewed to Date

Age	Caucasian	African-American	Other
35-44	110	14	5
45-54	229	19	2
55-64	261	19	2
65-74	212	11	2
75-84	80	5	0
Total	892	68	11

The overall participation rate for cases for the ongoing case-control study to date (as of 9/00) is approximately 40%. This participation rate includes individuals with incident breast cancer who were alive and eligible for the study, and whom we were able to contact. Numerous attempts have been made to maximize participation rate including working closely with area physicians who have been, on the average, quite supportive of our efforts (overall only 7% of the refusals are from the physician). We have worked extensively with local physicians to obtain good participation. We have worked closely with some of the breast surgeons and oncologists who see most of the breast cancer patients in order to attain good physician and patient participation.

It should be noted that we believe that our recruitment rate compares very favorably with other case-control studies conducted in the general population that require an extensive visit and blood drawing. Higher participation, at least in the U.S., has been reached only in studies where participants were interviewed by telephone, in hospitals or with minimal interview time or no blood drawing. The Department of Social and Preventive Medicine and the investigators involved in the proposed studies have long-term and successful experience in recruiting for epidemiological and clinical trial studies. We are part of the Western New York Vanguard Clinical Center of the Women's Health Initiative (WHI). The Western New York Center was the first nationwide (among 40 clinical centers) to meet recruitment goals for both the observational and clinical trial components of the study.

Table 2. Characteristics of participating and non-participating breast cancer cases completing a brief telephone questionnaire

	Participants (n=272)	Non-Participants (n=120)
Age (mean yrs ± sd)	58.8 (11.0)	64.8 (10.5)
Race (%)		
White	94	91
African American	5	8
Other	1	1
Education(mean yrs ± sd)	13.7 (2.3)	13.1 (2.4)
Alcohol Intake (drinks/mo)	3.8 (5.5)	3.1 (5.1)
Vegetable Intake	10 (4)	9 (4)

In order to assess the bias related to non-participation, we conducted a short interview with both those participating and those not participating, at the time of telephone contact, in order to characterize the non-respondents and compare them to those who did participate. All of those contacted were asked a small number of questions at the time of contact. Not all of these data have been entered; data that are available are presented here. Comparisons of responses of participating and non-participating breast cancer cases are shown in Table 2. In general, participants and non-participants completing this survey were similar. Participants were somewhat younger and more educated. There was little difference between the groups in either alcohol intake or vegetable intake.

Controls:

To date, we have interviewed 2,048 controls. We expect to interview another 227 controls before the interviewing is completed. This number will exceed what we agreed to in the statement of work. We have set this goal in order to have frequency matching of two controls for each case in the case controls study.

Controls under age 65 have been randomly selected from the list of those holding driver's licenses in Erie and Niagara counties; controls age 65 and over will have been randomly selected from the rolls of the Health Care Finance Administration. Excluded are women with a history of cancer other than non-melanoma skin cancer. Controls have been frequency-matched to cases on age, race, and county of residence.

Characteristics of controls interviewed to date are included in Table 3.

Table 3. Characteristics of Breast Cancer Cases Interviewed to Date

Age	Caucasian	African-American	Other
35-44	316	16	8
45-54	518	29	6
55-64	433	23	7
65-74	458	80	3
75-84	126	25	0
Total	1851	173	24

As for the cases, response rate is low, 37%. Again as for the cases, we have done a brief telephone interview to determine the characteristics of non-participants and participants. The available data are summarized in Table 4.

Table 4. Characteristics of participating and non-participating controls completing a brief telephone questionnaire

	Participants (n=1521)	Non-Participants (n=1123)
Age (mean yrs + sd)	58.4 (11.9)	61.4 (13.7)
Race (%)		
White	92	89
African American	7	9
Other	1	3
Education(mean yrs + sd)	13.7 (2.4)	12.8 (2.2)
Alcohol Intake (drinks/mo)	4.1 (5.4)	3.0 (4.8)
Vegetable Intake (servings/wk)	10 (4)	9 (4)

As for the cases, participants and non-participants completing this survey were very similar. Again, participating controls were somewhat younger and more educated. There was little difference between the groups in either alcohol intake or vegetable intake.

Task 4: Months 3-47: Ongoing data entry of the interview, maintenance of files from computer-assisted interview and entry of data from the sections of the interview completed by the participant.

All necessary arrangements for ongoing data entry of the interview, maintenance of files from the computer-assisted interview, and coding of data from the sections of the interview completed by hand by the participant are progressing in a timely fashion. Copies of the computer interview and self-administered questionnaires are included in the Appendix.

Task 5: Months 4-47: Maintenance of the biological specimen bank, processing of samples for immediate determinations and for storage, tracking of all samples, mapping of the freezer.

Procedures for the ongoing maintenance of the biological specimen bank ongoing. Means for tracking of samples and mapping of the freezer have been established and are progressing. The protocol for the storage system is included in the appendix.

To ensure standardization of specimens collected, all blood is drawn at the same time of the day (7:00AM-9:00AM). For pre-menopausal women, blood drawings are scheduled for the luteal phase of the cycle to reduce, to the extent possible, variation in hormone levels related to the menstrual cycle. The time of the blood draw is recorded for assessment of any variation in blood markers related to the time of the draw. 1,862 control and 520 case blood samples have been processed for immediate determinations and for long term storage.

Of the cases, 82% provide a blood sample; some cases are unable or unwilling to provide a blood sample because of treatment regimens. For controls, 92% consent to provide a blood specimen. All others are asked to provide a saliva sample and there is complete compliance with this request. There are 56 cases and 149 controls without any sample because at the start of the study we did not collect saliva samples. At the end of the study, we expect 884 cases and 1923 controls will have blood samples; 181 cases and 167 controls will have a saliva sample, obtained by the method of Lum and LeMarchand (2). Therefore we will be able to measure genotypes on a total of 1065 cases and 2090 controls.

We have published several papers recently regarding the issues of obtaining and storage of biological samples (3-6). Those manuscripts are included in the appendix.

Tasks 6: Months 25-48: Genetic analyses of samples: DNA extractions and determinations of genetic polymorphisms.

Blood clots for DNA extraction and subsequent genetic analysis have been removed from the freezer and shipped on dry ice to Dr. Peter Shields. We have sent 1744 samples: 560 from breast cancer cases, 560 pairs of controls, matched to cases, 2:1 and 60 randomly positioned blind duplicates. These samples represent most of the breast cancer cases whom we had interviewed for whom we have a blood sample at the time that the shipment was made during the summer. We have found that it is also possible to extract DNA from collected urine and saliva samples. At the conclusion of the study, we will also extract DNA samples from urine or saliva from those participants who gave consent but were either unable or unwilling to provide a blood specimen. DNA extraction is ongoing in Dr. Shields' lab. He has begun the ADH3 analyses and will continue with those.

Analysis for the genetic polymorphism in alcohol dehydrogenase 3 (ADH3) will be performed at this time for the DNA samples that have been sent to Dr. Shields' lab. Preliminary

results from that subset of the total sample have been analyzed to determine the modifying effects of ADH3 on the association between alcohol consumption and breast cancer risk; those results are given below.

Task 7: Months 25-48. Statistical analyses; preparation of variables from the interview and blood determination, all required analyses of the data for reports and presentations.

Preliminary statistical analyses are beginning. Final analysis will begin when interviewing has been completed. In the past year, preliminary data sets have been assembled and necessary work begun on managing the data, identifying outliers and implementing procedures for cleaning the data. Considerable work has been done on the alcohol data, the major focus of the interview. In addition, there has been considerable work done on the interview data regarding hormone use, both oral contraceptives and postmenopausal hormones. We have done work on the reports of family history of cancer, the information provided regarding vitamin usage and the information on the measures of anthropometry.

Task 8: Months 25-48. Preparation of publications reports and presentation of the data.

Because data collection is still underway, only one presentation has been made on these data. Results of the preliminary findings regarding ADH3, alcohol consumption and breast cancer risk were presented at the DoD Era of Hope meeting. The abstract and the tables from that presentation follow. These findings need to be considered very preliminary because they do not include the entire study sample. However, it is very interesting that we found very similar results to those of our previous study. It appears that there is an increase in risk associated with relatively low alcohol consumption in premenopausal women with the at risk genotype. When data collection is completed, we will continue these analyses with the full data set and examine the associations with different aspects of alcohol consumption (e.g., consumption at different periods of life, consumption of different beverages, patterns of drinking).

ALCOHOL CONSUMPTION, ALCOHOL DEHYDROGENASE GENOTYPE AND BREAST CANCER RISK

JL Freudenheim, P Muti, M Trevisan, R Browne, M Ram, D Vito, ED Bowman, R Goldman, P Shields

In a previous study, we found that the association between alcohol consumption and breast cancer risk was modified by a common variant in the gene for alcohol dehydrogenase (ADH_3). ADH_3 is rate-limiting, catalyzing the metabolism of alcohol to acetaldehyde, a probable carcinogen. We found a 3.5-fold increase in risk for premenopausal women with the apparently higher activity variant, ADH_3^{1-1}, and with higher alcohol consumption.

Presented here are preliminary findings of a new case-control study of alcohol and breast cancer where we tried to replicate these results with a more complete assessment of lifetime alcohol consumption. Women with incident, primary, histologically confirmed breast cancer were identified from pathology records in two counties in western New York. Controls < age 65 were randomly selected from lists of NY Driver's licenses, those $\geq$ 65 from lists of the Health Care Finance Administration. Cases who gave a blood sample (n=278) were matched on age, race and menopausal status with controls (n=484). Participants were interviewed regarding lifetime

alcohol consumption; questions included beverage-specific quantity, frequency, drink size, binge drinking, and consumption with meals or outside of meals. Genomic DNA was genotyped for the exon VIII ADH polymorphism by PCR followed by restriction enzyme digestion. Odd ratios (OR) and 95% confidence intervals (CI) were for four groups defined by alcohol consumption and ADH genotype, adjusted for other breast cancer risk factors. The referent was women with below median alcohol and either ADH_3^{2-2} or ADH_3^{1-2} genotype.

For postmenopausal women, we found no increase in risk for breast cancer in any of the groups. For premenopausal women, we found that for women with both higher alcohol consumption and the ADH_3^{1-1} genotype, there was an increase in risk (OR 2.60; 95% CI 0.93-7.23) relative to women with the other genotypes and lower alcohol consumption. Risk was not increased risk in the other two premenopausal groups. These preliminary results indicate that for premenopausal women, the increased risk associated with alcohol consumption may be limited to women with genetic susceptibility.

Table 5. Lifetime Alcohol Consumption and Risk of Breast Cancer in the Study Sample

Alcohol	Cases	Controls	Crude OR	Adj OR* (CI)
		Premenopausal		
Lower	25	58	1.00	1.00
Higher	30	50	1.39	1.41(0.68-2.91)
Total	55	108		
		Postmenopausal		
Lower	120	235	1.00	1.00
Higher	106	216	0.96	0.97(0.69-1.35)
Total	226	451		

*Odds ratios and 95% confidence intervals adjusted for age, education, age at first birth, age at menarche, parity, duration lactation, BMI, history of benign breast disease, vegetable intake, and fruit intake

Table 4. Characteristics of study sample by case and control status and alcohol dehydrogenase3 (ADH_3) genotype

Characteristics*	PREMENOPAUSAL WOMEN					
	Cases			Controls		
	ADH_3^{1-1}	ADH_3^{1-2}	ADH_3^{2-2}	ADH_3^{1-1}	ADH_3^{1-2}	ADH_3^{2-2}
N	27	20	8	38	53	13
Age(years)	44.9(6.1)	48.0(4.3)	44.0(3.7)	45.2(4.8)	45.7(4.6)	45.5(5.9)
Education(years)	14.3(2.7)	13.0(3.5)	13.8(2.2)	14.6(2.3)	14.6(2.1)	15.0(2.3)
Age at menarche(years)	12.3(1.4)	12.2(1.0)	12.3(1.8)	12.4(1.3)	12.7(1.7)	12.6(1.4)
Body Mass Index	28.8(7.9)	30.3(6.9)	27.4(6.6)	27.6(6.5)	27.4(5.6)	26.0(5.1)
Family History of Breast Cancer (%)	18.5	25.0	25.0	10.5	7.5	23.1
Duration lactation (months)	3.2(5.4)	5.5(10.5)	5.6(9.3)	8.1(12.2)	7.4(10.1)	12.1(12.4)
Parity	2.0(1.6)	1.9(1.2)	1.4(1.1)	2.0(1.5)	1.9(1.2)	2.7(1.3)
Age at first birth (years)	18.6(12.1)	21.4(10.7)	17.5(11.4)	21.2(11.9)	22.0(10.9)	23.0(7.6)
Lifetime alcohol (ounces)	1081(1315)	707(904)	680(623)	955 (1740)	1299(3096)	501(674)

POSTMENOPAUSAL WOMEN

N	73	122	28	141	182	59
Age (years)	62.2(9.4)	61.4(10.0)	64.8(6.9)	61.5(8.7)	62.1(9.7)	61.8(8.7)
Education (years)	13.2(2.3)	13.2(2.4)	13.4(2.9)	13.0(2.4)	13.1(2.4)	13.2(2.5)
Age at menarche (years)	12.6(1.8)	12.4(1.7)	12.1(1.2)	12.8 (1.9)	12.9(1.7)	12.8(1.7)
Body Mass Index	29.9(6.9)	28.7(6.4)	27.6(5.4)	28.9(6.3)	28.2(6.0)	28.8(6.9)
Family History of Breast Cancer (%)	13.7	16.4	17.9	12.8	18.1[a]	5.1[a]
Duration lactation (months)	3.8(8.2)	4.4(9.9)	3.5(6.6)	5.2(12.1)	5.0(9.7)	5.1(12.1)
Parity	2.2[b](1.9)	2.8(1.7)	3.5[b](1.8)	2.9(1.8)	2.8(2.0)	3.2(1.7)
Age at first birth (years)	17.7[c](11.3)	21.0[c](8.5)	22.2(5.9)	20.9(7.8)	21.4(9.1)	21.3(6.4)
Lifetime alcohol (ounces)	1378(2652)	1448(2918)	3293(7730)	2275(10669)	1409(3428)	975(2358)

*Values shown are mean (SD) except for family history of breast cancer which are percentages with positive history among either the cases or controls. Two-sided comparisons of means between the ADH_3 groups within cases or controls were computed by ANOVA; comparisons of categories were with the chi-square test. Those with the same letter are significantly different, $p<0.05$.

Table 6. Risk of Breast Cancer by ADH_3 Genotype

	Cases	Controls	Crude OR	Adjusted OR (CI)
		Premenopausal		
2-2	8	13	1.00	1.00
1-2	20	53	0.61	0.50(0.16-1.66)
1-1	27	38	1.15	0.95(0.30-3.01)
Total	55	104		
		Postmenopausal		
2-2	28	59	1.00	1.00
1-2	122	182	1.41	1.45(0.86-2.45)
1-1	73	141	1.09	1.09(0.63-1.89)
Total	223	382		

*Odds ratios and 95% confidence intervals adjusted for age, education, age at first birth, age at menarche, parity, duration lactation, BMI, history of benign breast disease, vegetable intake, fruit intake and alcohol intake.

Table 7. Risk of Breast Cancer by ADH_3 genotype and Lifetime Alcohol Consumption

	Cases	Controls	Crude OR	Adj OR* (CI)*
		Premenopausal		
ADH_3^{2-2} + ADH_3^{1-2}				
Lower alcohol	15	34	1.00	1.00
Higher alcohol	13	32	0.92	0.84(0.31-2.24)
ADH_3^{1-1}				
Lower alcohol	10	23	0.99	0.78(0.26-2.30)
Higher alcohol	17	15	2.57	2.60(0.93-7.23)
		Postmenopausal		
ADH_3^{2-2}+ADH_3^{1-2}				
Lower alcohol	76	133	1.00	1.00
Higher alcohol	74	108	1.20	1.18(0.77-1.80)
ADH_3^{1-1}				
Lower alcohol	42	72	1.02	0.95(0.58-1.55)
Higher alcohol	31	69	0.79	0.80(0.47-1.35)

*Odds ratios and 95% confidence intervals adjusted for age, education, age at first birth, age at menarche, parity, duration lactation, BMI, history of benign breast disease, vegetable intake, fruit intake and alcohol intake.

In addition to this presentation, we have submitted several grants to do further investigations enhancing the already collected data from this breast cancer case-control study. Two small grants have received funding, a third R01 was just submitted to the National Institutes of Health. The abstracts for each of those grants follow. The first, funded by the DoD, will examine residence at the time of birth and at menarche in relation to exposures from environmental contaminants and risk of breast cancer. This study will utilize a residential history that was part of the original interview with cases and controls. There will be additional work identifying sources of exposure during the relevant time period, and doing mapping to assess the relation between residence, exposure and risk. Additional analyses of several genetic factors will be included. The second study, funded by the National Cancer Institute, will examine residence in the period of life following menarche. Similar exposures and genetic factors will be examined. The third proposed study would include a focus on alcohol and the underlying mechanism for the association we see with risk. This grant would involve the collection of archived tumor blocks from the participants in our study. We propose to examine genetic factors, particular biomarkers in the tumor tissue as well as the interview data to examine two possible mechanisms to explain the observed associations of diet and breast cancer risk. Much of this study would include a particular focus on the mechanism for the observed alcohol association.

Environmental Exposures at Birth and at Menarche and Risk of Breast Cancer (funded by the DoD); Jo Freudenheim, P.I.

There is considerable evidence that environmental factors are important in the etiology of breast cancer. Breast cancer rates have changed markedly over time in genetically stable populations and there are important changes in rates for migrants from low risk to high-risk areas. Environmental factors related to industrialization may be important contributors to risk. Risk tends to be higher in more industrialized societies. There has been little study of proximity to industrial sites, to toxic waste sites and to other potentially toxic sources as potential risk factors. In particular, aromatic hydrocarbons such as benzene and some polycyclic aromatic hydrocarbons (PAHs) such as benzo(a)pyrene may be important in relation to risk. Further, genetic variation may alter the association of exposure with risk. There is evidence that benzene exposure is affected by variation in the gene for NQO1. Metabolism of PAHs is affected by genetic variation in detoxification systems, including GST M1-1, GST P1-1 and cytochrome P450 1A1 (cyp1A1). Recently attention has focused on the infant period, early childhood and menarche as potentially sensitive periods of exposure. Breast tissue cell division is particularly rapid and therefore may be more sensitive to environmental insults . There are few studies examining these time periods; none have focused on these exposures. We propose here to examine environmental exposures and gene-environment interactions at the time of birth and at menarche and subsequent risk of breast cancer.

We propose a population-based study to examine location of residence during these potentially sensitive time periods in relation to proximity to industrial sites, gasoline stations, toxic waste sites and heavily trafficked roadways as risk factors for subsequent disease. We expect that there will be an increase in risk for women who lived close to these sites during childhood, in particular, that point sources of benzene and PAHs will increase risk. Further, we wish to examine gene-environment interactions for these exposures experienced at birth and at menarche. The aims of the study are: 1) To investigate distance from steel mills, chemical factories, gasoline stations, toxic waste sites and other industrial sites of the residence of cases and controls at the time of birth and at menarche as risk factors for pre- and postmenopausal breast cancer, with control for appropriate confounders, and 2) To examine estimated exposure to benzene and to PAHs as risk factors for pre- and postmenopausal breast cancer, with control for the appropriate confounders. A secondary aim is: 3) To evaluate genetic susceptibility in relation to these exposures and breast cancer.

We will use data from an ongoing case-control study of breast cancer in Erie and Niagara Counties. We will collect residential histories, DNA for genotyping and data on other breast cancer risk factors for approximately 1000 cases of incident, primary, histologically-confirmed breast cancer

and more than 2000 controls, age 35-79, frequency-matched to cases on age, race and county. Addresses for the women at the time of their birth and at menarche will be geocoded using a Geographic Information System (GIS). Historical data will be collected regarding location of steel mills, chemical factories, gasoline stations, toxic waste sites and other industrial sites during the period 1918-80. Distance between these sites and the residences of cases and controls at the time of birth and at menarche will be calculated using the GIS. More than 75% of participants in the original study lived in these counties at the time of their births. Molecular analysis will be performed at the Laboratory for Human Carcinogenesis of the NCI for the polymorphisms in NQO1, GSTM1-1, GST P1-1 and cyp 1A1. We will calculate odds ratios and 95% confidence intervals for distance from each category of potential exposure and for an index of probable level of exposure to PAHs and to benzene. We will also examine risk within categories stratified on genotype. The proposed study will contribute to our understanding of the role of environmental exposures during infancy and menarche. This is a unique and cost effective opportunity to examine a hypothesis of potentially great public health importance in a relatively residentially stable population.

Breast Cancer Risk: Residential Environment and Genetics (funded by NCI); Jo Freudenheim, P.I.

There is evidence that environmental factors related to industrialization may be important in breast cancer etiology. There has been little study of proximity to potentially toxic industrial sites as breast cancer risk factors. We propose to conduct a case-control study to examine location of residence during adult life in relation to breast cancer risk. The aims of the study are: 1) To investigate distance from steel mills, chemical factories, and other industrial sites of the residence as risk factors. The time periods to be examined will be (1)the primary residence during the period between menarche and first pregnancy (if any, otherwise menopause) and (2) residence(s) 10 and 20 years ago; 2) To examine estimated exposure to benzene and to PAHs based on residential exposure during these time periods as risk factors. Secondary objectives are: 3) To examine genetic variability in metabolism by NQO1, GST M1-1, GST P1-1 and CYP 1A1in relation to these exposures and breast cancer risk; 4) To evaluate all adult residences in relation to distance from potentially important exposures (steel mills, chemical factories, etc.) and risk; 5) To examine estimated exposure to benzene and to PAHs during the entire adult life and risk. We will use data from an ongoing case-control study of breast cancer in Erie and Niagara Counties including approximately 1000 cases of incident, primary, histologically-confirmed breast cancer and more than 2000 controls, age 35-79, frequency-matched to cases. About 75% of participants in the original study lived in these counties at the time of their menarche. Addresses for the women at the time of their birth and at menarche will be geocoded using a Geographic Information System (GIS). Historical data will be collected regarding location of potentially important industrial sites. We will calculate odds ratios and 95% confidence intervals for distance from each category of potential exposure and for an index of probable level of exposure to PAHs and to benzene and we will examine risk within categories stratified on genotype. This is a unique and cost effective opportunity to examine a hypothesis of potentially great public health importance in a relatively residentially stable population.

Methylation and Oxidation in Breast Cancer Epidemiology (submitted to NCI), Jo Freudenheim, P.I.

There is considerable epidemiologic evidence that alcohol intake is related to risk of breast cancer and that intake of vegetables and fruits may reduce risk. Utilizing an existing case control study, we propose to examine two etiological mechanisms, one-carbon metabolism and/or oxidative stress and breast cancer. Our first aim is to examine the relation of elements related to one-carbon metabolism with risk. We propose a) to investigate reported intakes of relevant nutrients (alcohol, folate, methionine, vitamins B_6 and B_{12}) and risk; b) to investigate genetic variation in enzymes important in one-carbon metabolism (methylene tetrahydrofolate reductase (MTHFR),

methionine synthase (MS) and cystathione B-synthase (CBS)) in relation to risk and to investigate interaction of these genetic and dietary factors with risk; and c) to investigate the association of these dietary factors with p53 mutations and with hypermethylation of the p16 gene, the BRCA1 gene and the estrogen receptor gene in breast tumors. Our second aim is to examine elements related to oxidative stress and antioxidants with risk. We propose to a) examine the association of intake of total fruits and vegetables, and of vitamins C, E, A and carotenoids with risk; b) to examine the relation of genetic variation in an enzyme important in the control of oxidative balance (manganese superoxide dismutase ($MnSOD_2$)) and to examine interactions of this genetic factor with dietary factors; and c) to investigate the association between these dietary factors and p53 mutations. By combining information on intake, genetic susceptibility and tumor characteristics, it will be possible to make clearer inferences about the role of these two mechanisms in breast cancer etiology, with potentially important public health implications.

(6) Key Research Accomplishments

- Interview of 971 women with primary, histologically-confirmed breast cancer
- Interview of 2,048 controls, frequency matched to cases on age, race and county (76 controls were interviewed as part of this grant, the interviews for the remainder were funded under another grant).
- DNA extraction completed on 221 cases and 559 controls as well as for quality control samples. ADH3 measures completed for these participants. Samples for additional , more recently interviewed participants have been sent for DNA extraction. That extraction and the ADH3 analyses are ongoing.
- Data are being coded and entered for all the interviews. Ongoing is the examination of data for mistakes, outliers and inconsistencies.
- Preliminary analyses of ADH3 and risk of breast cancer have been presented at the DoD Era of Hope meeting. These very preliminary findings appear to indicate that there is a group of premenopausal women who are genetically susceptible to an increased risk of breast cancer associated with alcohol consumption. These findings will be investigated in more depth when the interviewing is finished.
- Two grants examining new hypotheses and utilizing this same data set have been funded. Another grant has been submitted that would focus on alcohol and diet and two potential underlying mechanisms of an observed association.

(7) Reportable Outcomes

1. Abstract presentation at DoD Era of Hope Meeting, ALCOHOL CONSUMPTION, ALCOHOL DEHYDROGENASE GENOTYPE AND BREAST CANCER RISK

2. Grants funded based on this research:
   - Environmental Exposures at Birth and at Menarche and Risk of Breast Cancer (funded by the DoD); Jo Freudenheim, P.I.
   - Breast Cancer Risk: Residential Environment and Genetics (funded by NCI); Jo Freudenheim, P.I.
3. Grants submitted based on this research:
   - Methylation and Oxidation in Breast Cancer Epidemiology (submitted to NCI), Jo Freudenheim, P.I.

(8) Conclusions

Because of the nature of epidemiologic studies, there are no interim results to report until the research has been finished. With out study we are attempting to elucidate the association between alcohol and breast cancer, examining a detailed and in-depth history of lifetime alcohol consumption including particular beverages, portions, drinking with or not with meals, pattern of drinking (i.e., differentiating occasional heavy drinking from consistent light drinking). We are also looking at how associations may differ by genetic susceptibility and by other risk factors including use of postmenopausal hormones, and by estrogen receptor status and histology for the cases. In that alcohol is one of the few easily modified risk factors that has been consistently identified for breast cancer, a clearer understanding of this risk factor is merited. In particular, if there are groups of women who are at particular risk for an effect of alcohol because of genetic susceptibility, this finding would have important public health implications.

(9) References

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APPENDICES

Computer interview

Self-administered Questionnaires

Protocol for Specimen Storage

Publications

COMPUTER INTERVIEW

PART III
INTERVIEWER ADMINISTERED SECTION

Before beginning interview, make certain that the following have been completed by clinical interviewer:

- ☐ Past Medical History reviewed
- ☐ 30 Day Medication Inventory reviewed
- ☐ Section I reviewed for completeness
- ☐ Height, weight, blood pressure and other measurement are completed

CHECKQa:
MAIN STUDY QUESTIONNAIRE - INTRODUCTORY SECTION

Do you want to start the interview for a new respondent or do you want to restart a previous interview?
Press "N" for new respondent
"R" to restart a previous interview

At any time, you can press ctrl/end to terminate an interview.

RETRY:
Type the respondent number to be restarted: ____
Press CTRL/END to exit the interview.

lognum:
Please enter the number of the interview to begin and then press enter: ____

gender:
Please enter the gender of the respondent. Is the respondent female or male?
1. *Female*
2. *Male*

MONTH:
Please enter the current month. Use three letter abbreviation: ____
JAN, FEB, MAR, APR, MAY, JUN, JUL, AUG, SEP, OCT, NOV, DEC

iwinit:
Please enter your interviewer number: ____

INTRODUCTION:
Please have a seat at the desk and we will begin the interview. During the interview, I will be asking you some lifestyle and medical questions. The topics I will be asking you about include physical activity level, smoking, alcohol consumption, and family history of disease. Because it is important to find out about your lifestyle at different ages, some of the questions will be repeated for different periods of your life. Some of these questions will ask you to approximate the size of beverage containers. Pictures will be available to help you in answering these questions.

I want to emphasize again that all answers you give as part of this study are confidential and will not be discussed with anyone. When reports are prepared, only group answers will be used – responses from individuals are never reported. So, please be as honest as you can be in answering these questions. If you do not know the exact answer, please make your best guess.

As you can see, your responses will be entered into this computer. As we go along, I may pause between questions because it takes time for the computer to compile some of the information you are giving me.

If you need a break at any time during the interview, just let me know. Are you ready to begin now?

PRESS ANY KEY TO CONTINUE...

agebirth:
We will begin with some background questions we ask everyone. When were you born? *Enter date of birth (MM/DD/YY).*
(Respondent must have been born between 1913 and 1963):
__/__/__

intstop:
End of main study introductory questionnaire for this respondent.....Press any key to continue.....

CURRENT PHYSICAL ACTIVITY

1. Now, we would like to know about your physical activity during the past 7 days. But first, let me ask you about your sleep habits. On the average, how many hours did you sleep each night during the last five weekday nights, that is Sunday through Thursday night?

   ____ hours/night
   88 Don't Know
   99 Refused

2. On the average, how many hours did you sleep each night last Friday and Saturday nights?

   ____ hours/night
   88 Don't Know
   99 Refused

Now I am going to ask you about your physical activity during the past 7 days, that is, the last 5 weekdays, and last weekend, Saturday and Sunday. We are not going to talk about light activities such as slow walking, light housework, or unstrenuous sports such as bowling, archery, or softball. Please look at this card which shows some examples of what we consider MODERATE, HARD, and VERY HARD ACTIVITIES. *(Hand card allow time for participant to read.)* People engage in many other types of activities and if you are not sure where one of your activities fits, please ask me about it.

Interviewer: If you don't know how to classify any activities, type a <CTRL> Note where you think the activity may belong.

3. First, let's consider moderate activities. What activities did you do and how many total hours did you spend during the **last 5 weekdays** doing these moderate activities or others like them? Please tell me to the nearest half hour.

   Occupational____._
   Household____._
   Sports____._
   88 Don't Know
   99 Refused

(CARD: 1 A-B MODERATE ACTIVITY

OCCUPATIONAL TASKS:
Delivering mail or patrolling on foot
House painting
Truck driving (making deliveries, lifting and carrying light objects)
HOUSEHOLD ACTIVITIES:
Raking the lawn
Sweeping and mopping
Mowing the lawn with a power mower
Cleaning windows
SPORTS ACTIVITIES (ACTUAL PLAYING TIME):
Volleyball
Ping-pong
Brisk walking for pleasure or to work (20 min/mile or 3 miles/hr)
Golf, walking and pulling or carrying clubs
Calisthenic exercises)

4. Last **Saturday and Sunday**, how many hours did you spend on moderate activities? (Probe: Can you think of any other sports, job, or household activities that would fit into this category?)

Occupational____._
Household____._
Sports____._
88 Don't Know
99 Refused

(CARD: MODERATE ACTIVITY
OCCUPATIONAL TASKS:
Delivering mail or patrolling on foot
House painting
Truck driving (making deliveries, lifting and carrying light objects)
HOUSEHOLD ACTIVITIES:
Raking the lawn
Sweeping and mopping
Mowing the lawn with a power mower
Cleaning windows
SPORTS ACTIVITIES (ACTUAL PLAYING TIME):
Volleyball
Ping-pong
Brisk walking for pleasure or to work (20 min/mile or 3 miles/hr)
Golf, walking and pulling or carrying clubs
Calisthenic exercises)

5. Now let's look at hard activities. *(Show Card: Hard Activity)* What activities did you do and how many total hours did you

spend during these hard activities or others like them? Please tell me to the nearest half hour.

Occupational____._
Household____._
Sports____._
88 Don't Know
99 Refused

(CARD: HARD ACTIVITY
OCCUPATIONAL TASKS:
Heavy carpentry
Construction work, doing physical labor
HOUSEHOLD ACTIVITIES:
Scrubbing floors
SPORTS ACTIVITIES (ACTUAL PLAYING TIME):
Tennis doubles
Disco, square, or folk dancing)

6. Last **Saturday and Sunday**, how many hours did you spend on hard activities? (probe: Can you think of any other sports, job, or household activities that would fit into this category?)

Occupational____._
Household____._
Sports____._
88 Don't Know
99 Refused

(CARD: HARD ACTIVITY
OCCUPATIONAL TASKS:
Heavy carpentry
Construction work, doing physical labor
HOUSEHOLD ACTIVITIES:
Scrubbing floors
SPORTS ACTIVITIES (ACTUAL PLAYING TIME):
Tennis doubles
Disco, square, or folk dancing)

7. Now let's look at very hard activities. *(Show Card: Very Hard Activities)* What activities did you do and how many total hours did you spend during the **last 5 weekdays** doing these very hard activities or others like them? Please tell me to the nearest half hour.

Occupational____._
Sports____._
88 Don't Know
99 Refused

(CARD: VERY HARD ACTIVITY
OCCUPATIONAL TASKS:
Very hard physical labor, digging or chopping with heavy tools
Carrying heavy loads such as bricks or lumber
SPORTS ACTIVITIES (ACTUAL PLAYING TIME):
Jogging or swimming
Singles tennis
Racquetball
Soccer)

8. Last **Saturday and Sunday**, how many hours did you spend on very hard activities? (Probe: Can you think of any other sports, job, or household activities that would fit into this category?)
Occupational____._
Sports____._
88 Don't Know
99 Refused

(CARD: VERY HARD ACTIVITY
OCCUPATIONAL TASKS:
Very hard physical labor, digging or chopping with heavy tools
Carrying heavy loads such as bricks or lumber
SPORTS ACTIVITIES (ACTUAL PLAYING TIME):
Jogging or swimming
Singles tennis
Racquetball
Soccer)

9. Compared with your physical activity <u>OVER THE PAST 3 MONTHS</u>, was last week's physical activity more, less or about the same?
A. More
B. Less
C. About the same
88 Don't Know
99 Refused

Do pen and paper version of response sheet for stanford questionnaire. Double check that individual activities sum to previously reported hours. Hit enter for "totals" screen to appear...

10. Now we will review the answers you've just given. During the last (5 weekdays/ 2 weekend days) you told me you spent __ hours doing (moderate/hard/very hard) (occupational/household/sports) tasks. Can you please list them for me?

ACTIVITIES LAST 5 WEEKDAYS

MODERATE ACTIVITIES

OCCUPATIONAL	999.99
HOUSEHOLD	999.99
SPORTS	999.99
TOTAL:	999.99

HARD ACTIVITIES

OCCUPATIONAL	999.99
HOUSEHOLD	999.99
SPORTS	999.99
TOTAL:	999.99

VERY HARD ACTIVITIES

OCCUPATIONAL	999.99
HOUSEHOLD	999.99
SPORTS	999.99
TOTAL:	999.99

ACTIVITIES ON SATURDAY & SUNDAY

MODERATE ACTIVITIES

OCCUPATIONAL	999.99
HOUSEHOLD	999.99
SPORTS	999.99
TOTAL:	999.99

HARD ACTIVITIES

OCCUPATIONAL	999.99
HOUSEHOLD	999.99
SPORTS	999.99
TOTAL:	999.99

VERY HARD ACTIVITIES

OCCUPATIONAL	999.99
HOUSEHOLD	999.99
SPORTS	999.99
TOTAL:	999.99

Verify above totals with written section, does everything check out okay? (Y/N)

CURRENT DRINKING PATTERNS ASSESSMENT
12 months that ended one year ago
Past 30 days

In this portion of the interview, we want to assess your exposure to alcohol.

1. In your entire life, have you had at least 12 drinks of any kind of alcoholic beverage? As alcoholic beverages, we include beer, wine coolers, wine, and liquor.

   1 Yes (Skip to Q5.)
   2 No
   9 Refused

2. How many alcoholic drinks would you say you have had in your entire lifetime?

   Enter number of drinks _____ _____

   99 Refused

If respondent answers 12 or more, press Escape to back up and re-ask Q1. Computer will not accept an answer of 12 or more.

Show Card I-1

3. ***If 11 or less, ask:***
   Please look at this list of reasons for not drinking and tell me which ones are your reasons for not drinking. Choose as many as apply.

   Enter all that are given.

   01 Don't socialize very much
   02 Don't care for it or dislike it
   03 Am an alcoholic
   04 Thought I might become an alcoholic
   05 Had problems with my drinking
   06 Have a responsibility to my family
   07 Family member an alcoholic or problem drinker
   08 Medical or health reasons
   09 Religious or moral reasons
   10 Brought up not to drink
   11 Makes me sick

12 Can't control my drinking
13 Costs too much or can't afford it
14 Dieting or too fattening
15 Other
98 Don't know/Not sure
99 Refused

Anything else?

Show Card I-1

4. Of the reasons you have just told me, which is your most important reason for not drinking?

Enter code ____ ____ ***(Skip respondent out of alcohol section.)***

99 Refused

5. How about during a one-year period? Did you ever have at least 12 drinks during a one-year period in your life?

1 Yes ***(Skip to Q8.)***
2 No
9 Refused

Show Card I-1

6. Please look at this list and tell me which ones are your reasons for not drinking frequently. Choose as many as apply.

Enter all that are given.

01 Don't socialize very much
02 Don't care for it or dislike it
03 Am an alcoholic
04 Thought I might become an alcoholic
05 Had problems with my drinking
06 Have a responsibility to my family
07 Family member an alcoholic or problem drinker
08 Medical or health reasons
09 Religious or moral reasons
10 Brought up not to drink
11 Makes me sick
12 Can't control my drinking

13 Costs too much or can't afford it
14 Dieting or too fattening
15 Other
98 Don't know/Not sure
99 Refused

Anything else?

7. Of the reasons you have just told me, which is your most important reason for not drinking frequently?

Enter code ____ ____ ***(Skip respondent out of alcohol section.)***

99 Refused

8. When was the last time you had a drink of any kind of alcoholic beverage?

1 Within the past 30 days
2 More than one month ago, but within the past 24 months
(Continue Q9 - Q41, then go to CLDH.)
3 More than 24 months ago ***(Skip to CLDH.)***
9 Refused

Show Card I-2

9. We are interested, first, in what you were drinking during the 12 months that ended one year ago, that is from _____ 1994 to _____ 1995. ***Use Timeline to illustrate***. During that period, how often did you drink some kind of alcoholic beverage?

1 Less than once a month or never
(If Q8=1, Skip to 30 days, Q42.)
(If Q8=2, Skip to CLDH.)
2 Once a month
3 Two days a month
4 Three days a month
5 Once a week or more often
9 Refused (Skip to 30 days, Q42.)

Show Card I-3

10. Which of the major beverages, beer, wine cooler, wine, and liquor, did you drink on a regular basis, that is an average of at least once a month for a period of at least six months, from ____ 1994 to ____ 1995? ***Check all that apply. Card also includes examples of specific types of beer, wine, and liquor to help trigger respondents' memories of their alcohol intake.***

Beer?

Wine Cooler?

Wine?

Liquor?

11. Beer comes in different sizes. What size beer did you usually have from ____ 1994 to ____ 1995? ***(Refer to models first; show photo 1 if necessary.)***

Glasses:

X. 2 oz
A. 4 oz
B. 6 oz
C. 8 oz
D. 10 oz
E. 12 oz
F. 14 oz

Bottles/Cans:

H. 7.5 oz (split)
J. 12 oz (regular)
L. 16 oz (pounder)
M. 20 - 25 oz (tall boy)
O. 32 oz (quart)
P. 40 oz (40 bo)

1 Other
9 Refused

Type the "Other" size respondent reported.
Press enter twice when you are finished.

12. Wine coolers come in different sizes. What size wine cooler did you usually have from ____ 1994 to ____ 1995? ***(Refer to models first; show photo 2 if necessary.)***

Glasses:

X. 2 oz
A. 4 oz
B. 6 oz
C. 8 oz
D. 10 oz
E. 12 oz
F. 14 oz

Bottles:

J. 12 oz
N. Fifth
O. Liter
Q. 2 liter

1 Other
9 Refused

Type the "Other" size respondent reported.
Press enter twice when you are finished.

13. People define a drink of wine in different ways. When you drank wine from ____ 1994 to ____ 1995, how much wine was in your usual drink?***(Refer to models first; show photo 3 if necessary.)***

Glasses:

X. 2 oz
A. 4 oz
B. 6 oz
C. 8 oz
D. 10 oz
E. 12 oz
F. 14 oz

Bottles/Carafes:

K. Half a fifth
L. Pint, 1/2 liter, 1/2 liter carafe
N. Fifth, standard bottle
O. Quart, liter, liter carafe

1 Other
9 Refused

Type the "Other" size respondent reported.
Press enter twice when you are finished.

14. People differ in the amount of liquor they have in their drinks. How much liquor did you have in your usual drink from ____ 1994 to ____ 1995?
(Refer to models first; show photo 4 if necessary.)

Glasses:

X. 2 oz
A. 4 oz
B. 6 oz
C. 8 oz
D. 10 oz
E. 12 oz
F. 14 oz

Bottles:

G. 50 ML
I. Half a pint
K. Half a fifth
L. Pint, 1/2 liter
N. Fifth, standard bottle
O. Quart, liter

Shot Glasses:

R. 1.0 oz
S. 1.25 oz

1 Other
9 Refused

Type the "Other" size respondent reported.
Press enter twice when you are finished.

The following question is asked of respondents who reported drinking at least once a month but less often than once a week during the period from ____ 1994 to ____ 1995.
(Ask only if Q9 = 2, 3, or 4. If Q9 = 5, Skip to Q16.)

15. On days when you drank alcohol during that period from ____ 1994 to ____ 1995, how many drinks did you usually have?

Enter number of drinks. ____ ____ ***(Skip to Q32.)***

99 Refused

16. Now we want to know about your drinking patterns. You may find it easier to remember if you think back to your activities in a typical month

during that period from ____ 1994 to ____ 1995. We will ask about drinking on weekends and weekdays.

Let's start with Friday. Think of what you usually did during that time period on Fridays, that is, did you go to work, stay home, go out, have friends over, whatever?

Let respondent think but do not record; ask:

On Fridays during a typical month, how often did you drink - every Friday, three Fridays, two Fridays, one Friday, or hardly ever on Fridays?

4 Every Friday
3 Three Fridays
2 Two Fridays
1 One Friday
0 Hardly ever on Fridays (Skip to Q19.)
9 Refused

17. How many drinks would you usually have on a Friday?

Enter number of drinks. ____ ____

99 Refused

18. What did you usually drink on Fridays? ***Read beverages with a 1 next to them. Select these beverages on list below.***

Beer
Wine coolers
Wine
Liquor

19. Now think about what you did on a typical Saturday during that period from ____ 1994 to ____ 1995. How many Saturdays during a typical month would you have something to drink?

4 Every Saturday
3 Three Saturdays
2 Two Saturdays
1 One Saturday
0 Hardly ever on Saturdays (Skip to Q22.)
9 Refused

20. How many drinks would you usually have on a Saturday?

Enter number of drinks. _____ _____

99 Refused

21. What did you usually drink on Saturdays? ***Read beverages with a 1 next to them. Select these beverages on list below.***

Beer
Wine coolers
Wine
Liquor

22. Now think about what you did on a typical Sunday during that period from ____ 1994 to ____ 1995. How many Sundays during a typical month would you have something to drink?

4 Every Sunday
3 Three Sundays
2 Two Sundays
1 One Sunday
0 Hardly ever on Sundays (Skip to Q25.)
9 Refused

23. How many drinks would you usually have on a Sunday?

Enter number of drinks. ____ ____

99 Refused

24. What did you usually drink on Sundays? ***Read beverages with a 1 next to them. Select these beverag***

Beer
Wine coolers
Wine
Liquor

25. Now think about the four weekdays, Monday through Thursday. During that period from ____ 1994 to ____ 1995, how often during a typical month would you usually drink on a weekday?

Show Card I-4

1	All four weekdays, every week	(16 weekdays)	
2	Three weekdays, every week	(12 weekdays)	
3	Two weekdays, every week	(8 weekdays)	
4	One weekday, every week	(4 weekdays)	
5	One to three weekdays a month	(1-3 weekdays)	
6	Hardly ever on weekdays	(0 weekdays)	***(Skip to Q28, or Q30.)***

7 Some other pattern. Try to fit respondent's frequency of weekday drinking during a typical month into codes above, i.e. 16 days a month, 12 days a month, etc. or code 7 and use Control-Note to record unusual frequency pattern reported.

9 Refused

26. How many drinks would you usually have on a weekday?

Enter number of drinks. _____ _____

99 Refused

27. What did you usually drink on weekdays? ***Read beverages with a 1 next to them. Select these beverages on list below.***

Beer
Wine coolers
Wine
Liquor

28. ***If respondent has answered "hardly ever" to drinking on all seven days of the week, the computer will call up Q28 and Q29:***

About how many days a week, on average, did you drink alcohol during that period from ____ 1994 to ____ 1995?

Enter number of days. _____

9 Refused

29. On those days, how many drinks on average did you usually have?

Enter number of drinks. _____ _____

99 Refused

Asked of respondents who reported drinking a beverage at least once a month, but did not report it as part of their weekly drinking pattern:

30. Let's see, you told me you drank (beer/wine cooler/wine/liquor) at least once a month from ____ 1994 to ____ 1995, but it didn't show up in your usual drinking pattern. About how many days a month did you drink (beer/wine cooler/wine/liquor) during that period?

    Enter number of days. _____ _____

    99 Refused

31. On a day when you drank (beer/wine cooler/wine/liquor) during that period, how many drinks of (beer/wine cooler/wine/liquor) did you have?

    Enter number of drinks. _____ _____

    99 Refused

32. Now think about that entire period from ____ 1994 to ____ 1995. Were there days when you had more than your usual?

    1 Yes
    2 No (Skip to Q35.)
    9 Refused

33. What did you typically drink on a day when you had more than your usual?

    Record number of beers and select unit on next screen. R's usual beer size is . Record usual unless another size is reported. Refer to models first; show photo 1 if necessary.

    Glasses:
    X. 2 oz
    A. 4 oz
    B. 6 oz
    C. 8 oz
    D. 10 oz
    E. 12 oz
    F. 14 oz

    Bottles/Cans:
    H. 7.5 oz (split)
    J. 12 oz (regular)

L. 16 oz (pounder)
M. 20 - 25 oz (tall boy)
O. 32 oz (quart)
P. 40 oz (40 bo)

1 Other
2 Pitcher (60 oz)
3 Six pack
4 Half case (12 regular beers: 12-pack, "chill")
5 Case (24 regular beers)
9 Refused

Type the "Other" size respondent reported.
Press enter twice when you are finished.

Record number of wine coolers and select unit on next screen. R's usual wine cooler size is . Record usual unless another size is reported. Refer to models first; show photo 2 if necessary.

Glasses:
X. 2 oz
A. 4 oz
B. 6 oz
C. 8 oz
D. 10 oz
E. 12 oz
F. 14 oz
Bottles:
J. 12 oz
N. Fifth
O. Liter
Q. 2 liter

1 Other
9 Refused

Type the "Other" size respondent reported.
Press enter twice when you are finished.

Record number of drinks of wine and select unit on next screen. R's usual wine size is . Record usual unless another size is reported. Refer to models first; show photo 3 if necessary.

Glasses:

X. 2 oz
A. 4 oz
B. 6 oz
C. 8 oz
D. 10 oz
E. 12 oz
F. 14 oz

Bottles/Carafes:
K. Half a fifth
L. Pint, 1/2 liter, 1/2 liter carafe
N. Fifth, standard bottle
O. Quart, liter, liter carafe
P. Wine Magnum (1.5 liters)

1 Other
2 Pitcher (60 oz)
6 Half gallon
7 Gallon
9 Refused

Type the "Other" size respondent reported.
Press enter twice when you are finished.

Record number of drinks of liquor and select unit on next screen. R's usual liquor size is . Record usual unless another size is reported. Refer to models first; show photo 4 if necessary.

Glasses:
X. 2 oz
A. 4 oz
B. 6 oz
C. 8 oz
D. 10 oz
E. 12 oz
F. 14 oz

Bottles:
G. 50 ML
I. Half a pint
K. Half a fifth
L. Pint, 1/2 liter
N. Fifth, standard bottle
O. Quart, liter
P. Liquor Magnum (1.75 liters)

Shot Glasses:
R. 1.0 oz
S. 1.25 oz

1 Other
9 Refused

Type the "Other" size respondent reported.
Press enter twice when you are finished.

Show Card I-5

34. How often would you drink more than usual?

1 Once a week or more
2 Three days a month
3 Two days a month
4 Once a month
5 7 to 11 days a year
6 3 to 6 days a year
7 2 days a year
8 Once a year
9 Refused

Show Cards I-6A and I-6B

35. Now think about all the times you had alcohol to drink during the 12 month period from ____ 1994 to ____ 1995. Out of 10 times when you drank alcohol, how often did you drink with a meal, how often did you drink while snacking, and how often did you have something to drink without eating anything?

Multiply response by 10 and record: 1 = 10, 2 = 20, etc. If respondent says less than one, record as 5 and adjust numbers accordingly. They must add to 100. If respondent refuses to answer, enter 999 instead of 100.

Drank with meals
Drank while snacking
Drank without eating anything

Show Cards I-7A and I-7B

36. Of all the beers that you drank from ____ 1994 to ____ 1995, how many out

of 10 were (***each type of beer***)?

Multiply response by 10 and record: 1 = 10, 2 = 20, etc. If respondent says less than one, record as 5 and adjust numbers accordingly. They must add to 100. If respondent refuses to answer, enter 999 instead of 100.

Show Cards I-7A and I-7B

37. Out of every ten drinks of wine you had during that period, how many out of 10 were (***each type of wine***)?

Multiply response by 10 and record: 1 = 10, 2 = 20, etc. If respondent says less than one, record as 5 and adjust numbers accordingly. They must add to 100. If respondent refuses to answer, enter 999 instead of 100.

Show Cards I-7A and I-7B

38. Out of every ten drinks of liquor you had during that period, how many out of 10 were (***each type of liquor***)?

Multiply response by 10 and record: 1 = 10, 2 = 20, etc. If respondent says less than one, record as 5 and adjust numbers accordingly. They must add to 100. If respondent refuses to answer, enter 999 instead of 100.

Q39 asked of respondents who drank more than one major beverage type.

Show Cards I-7A and I-7B

39. Again thinking of all the major beverage types you had to drink from ____ 1994 to ____ 1995, out of every ten drinks that you had during that period how many were (***major beverages as reported in Q10***)?

Multiply response by 10 and record: 1 = 10, 2 = 20, etc. If respondent says less than one, record as 5 and adjust numbers accordingly. They must add to 100. If respondent refuses to answer, enter 999 instead of 100.

40. During this period, did you ever drink enough to get drunk or very high, that is, your speech was slurred or you were unsteady on your feet?

1 Yes
2 No (Skip to Q42.)
9 Refused (Skip to Q42.)

Show Card I-8

41. How often did you drink enough to get drunk or very high?

1 5 days or more a week
2 3 to 4 days a week
3 1 to 2 days a week
4 1 to 3 days a month
5 7 to 11 days a year
6 3 to 6 days a year
7 2 days a year
8 Once a year
9 Refused

42. Now I need to ask more specific questions about drinking during the past 30 days.

Press any key to continue.

Show Card I-9

We are interested in what you were drinking during the past 30 days. During that period, how often did you drink some kind of alcoholic beverage?

1 Once a week or more
2 Three days in the past 30 days
3 Two days in the past 30 days
4 Once in the past 30 days
5 Not at all in the past 30 days ***(Skip to CLDH.)***
9 Refused

Show Card I-10

43. Which of the major beverages listed: beer, wine cooler, wine, liquor, did you drink at least once in the past 30 days? ***Check all that apply. Card also includes examples of specific types of beer, wine, and liquor to help trigger respondents' memories of their alcohol intake.***

Beer?

Wine Cooler?

Wine?

Liquor?

Questions about drink size for beverages reported in Q43. Program should prompt if drink size was reported for period 12 to 24 months ago.

44. Your previous (beer/wine cooler/wine/liquor) size was ___. Did it change in the past 30 days?

    1 Yes
    2 No (Enter previous drink size shown above.)
    9 Refused

What size (beer/wine cooler/wine/liquor) did you drink during the past 30 days? ***(Refer to models first; show photo 1/2/3/4 if necessary.)***

If drink size was not reported previously, program will ask:

45. (Beer/wine cooler/wine/liquor) comes in different sizes. What size (beer/wine cooler/wine/ liquor) did you usually have during the past 30 days? ***(Refer to models first; show photo 1/2/3/4 if necessary.)***

BEER:

Glasses:
    X. 2 oz
    A. 4 oz
    B. 6 oz
    C. 8 oz
    D. 10 oz
    E. 12 oz
    F. 14 oz

Bottles/Cans:
    H. 7.5 oz (split)
    J. 12 oz (regular)
    L. 16 oz (pounder)
    M. 20 - 25 oz (tall boy)
    O. 32 oz (quart)
    P. 40 oz (40 bo)

    1 Other
    9 Refused

Type the "Other" size respondent reported.
Press enter twice when you are finished.

WINE COOLER:

Glasses:

X. 2 oz
A. 4 oz
B. 6 oz
C. 8 oz
D. 10 oz
E. 12 oz
F. 14 oz

Bottles:

J. 12 oz
N. Fifth
O. Liter
Q. 2 liter

1 Other
9 Refused

Type the "Other" size respondent reported.
Press enter twice when you are finished.

WINE:

Glasses:

X. 2 oz
A. 4 oz
B. 6 oz
C. 8 oz
D. 10 oz
E. 12 oz
F. 14 oz

Bottles/Carafes:

K. Half a fifth
L. Pint, 1/2 liter, 1/2 liter carafe
N. Fifth, standard bottle
O. Quart, liter, liter carafe

1 Other
9 Refused

Type the "Other" size respondent reported.
Press enter twice when you are finished.
LIQUOR:

Glasses:

X. 2 oz
A. 4 oz
B. 6 oz
C. 8 oz
D. 10 oz
E. 12 oz
F. 14 oz

Bottles:

G. 50 ML
I. Half a pint
K. Half a fifth
L. Pint, 1/2 liter
N. Fifth, standard bottle
O. Quart, liter

Shot Glasses:

R. 1.0 oz
S. 1.25 oz

1 Other
9 Refused

Type the "Other" size respondent reported.
Press enter twice when you are finished.

The following question is asked of respondents who reported drinking less than once a week during the past 30 days (Q42=2,3 or 4).

46. On days when you drank alcohol during the past 30 days, how many drinks did you usually have?

Enter number of drinks. ____ ____ ***(Skip to Q63.)***

99 Refused

Now we want to know about your drinking patterns during the past 30 days. We will ask about drinking on weekends and weekdays.

Press any key to continue.

47. Let's start with Friday. On Fridays during the past 30 days, how often did you drink- every Friday, three Fridays, two Fridays or hardly ever on Fridays?

4 Every Friday
3 Three Fridays
2 Two Fridays
1 One Friday
0 Hardly ever on Friday (Skip to Q50.)
9 Refused

48. How many drinks would you usually have on a Friday?

Enter number of drinks. ____ ____

99 Refused

49. What did you usually drink on Fridays? ***Read beverages with a 1 next to them. Select these beverages on list below.***

Beer
Wine coolers
Wine
Liquor

50. Now think about what you did on a typical Saturday during the past 30 days. How many Saturdays did you have something to drink?

4 Every Saturday
3 Three Saturdays
2 Two Saturdays
1 One Saturday
0 Hardly ever on Saturdays (Skip to Q53.)
9 Refused

51. How many drinks would you usually have on a Saturday?

Enter number of drinks. ____ ____

99 Refused

52. What did you usually drink on Saturdays? ***Read beverages with a 1 next to them. Select these beverages on list below.***

Beer
Wine coolers
Wine
Liquor

53. Now think about what you did on a typical Sunday during the past 30 days. How many Sundays did you have something to drink?

4 Every Sunday
3 Three Sundays
2 Two Sundays
1 One Sunday
0 Hardly ever on Sundays (Skip to Q56.)
9 Refused

54. How many drinks would you usually have on a Sunday?

Enter number of drinks. ____ ____

99 Refused

55. What did you usually drink on Sundays? ***Read beverages with a 1 next to them. Select these beverages on list below.***

Beer
Wine coolers
Wine
Liquor

Show Card I-11

56. Now think about the four weekdays, Monday through Thursday. On how many weekdays did you usually have something to drink- all four days, three days out of four, two days, one day, or hardly ever on weekdays in the past 30 days?

1	***All four weekdays, every week***	***(16 weekdays)***
2	***Three weekdays, every week***	***(12 weekdays)***
3	***Two weekdays, every week***	***(8 weekdays)***
4	***One weekday, every week***	***(4 weekdays)***
5	***One to three weekdays a month***	***(1-3 weekdays)***
6	***Hardly ever on weekdays***	***(0 weekdays) (Skip to Q59,***

or Q61.)

7 Some other pattern. Try to fit respondent's frequency of weekday drinking during the past 30 days into codes above, i.e. 16 days a month, 12 days a month, etc. or code 7 and use Control-Note to record unusual frequency pattern reported.
9 Refused

57. How many drinks would you usually have on a weekday?

Enter number of drinks. _____ _____

99 Refused

58. What did you usually drink on weekdays? ***Read beverages with a 1 next to them. Select these beverages on list below.***

Beer
Wine coolers
Wine
Liquor

59. ***If respondent has answered "hardly ever" to drinking on all seven days of the week, the computer will call up Q59 and Q60:***

About how many days a week, on average, did you drink alcohol during the past 30 days?

Enter number of days. _____

9 Refused

60. On those days, how many drinks on average did you usually have?

Enter number of drinks. _____ _____

99 Refused

Asked of respondents who reported drinking a beverage at least once during the past 30 days, but did not report it as part of their weekly drinking pattern.

61. Let's see, you told me you drank (beer/wine cooler/wine/liquor) at least once in the past 30 days, but it didn't show up in your usual drinking pattern.

About how many days did you drink (beer/wine cooler/wine/liquor) in the past 30 days?

Enter number of days. _____ ____

99 Refused

62. On a day that you drank (beer/wine cooler/wine/liquor) during that period, how many drinks of (beer/wine cooler/wine/liquor) did you have?

Enter number of drinks. _____ ____

99 Refused

63. Now think about the past 30 days. Were there days when you had more than your usual?

1 Yes
2 No (Skip to Q66.)
9 Refused

64. What did you typically drink on a day when you had more than usual?
Record number of beers and select unit on next screen. R's usual beer size is . Record usual unless another size is reported. Refer to models first; show photo 1 if necessary.

Glasses:

X. 2 oz
A. 4 oz
B. 6 oz
C. 8 oz
D. 10 oz
E. 12 oz
F. 14 oz

Bottles/Cans:

H. 7.5 oz (split)
J. 12 oz (regular)
L. 16 oz (pounder)
M. 20 - 25 oz (tall boy)
O. 32 oz (quart)
P. 40 oz (40 bo)

1 Other
2 Pitcher (60 oz)

3 Six pack
4 Half case (12 regular beers: 12-pack, "chill")
5 Case (24 regular beers)
9 Refused

Type the "Other" size respondent reported.
Press enter twice when you are finished.

Record number of wine coolers and select unit on next screen. R's usual wine cooler size is . Record usual unless another size is reported. Refer to models first; show photo 2 if necessary.

Glasses:
X. 2 oz
A. 4 oz
B. 6 oz
C. 8 oz
D. 10 oz
E. 12 oz
F. 14 oz

Bottles:
J. 12 oz
N. Fifth
O. Liter
Q. 2 liter

1 Other
9 Refused

Type the "Other" size respondent reported.
Press enter twice when you are finished.

Record number of drinks of wine and select unit on next screen. R's usual wine size is . Record usual unless another size is reported. Refer to models first; show photo 3 if necessary.

Glasses:
X. 2 oz
A. 4 oz
B. 6 oz
C. 8 oz
D. 10 oz
E. 12 oz

F. 14 oz

Bottles/Carafes:

K. Half a fifth
L. Pint, 1/2 liter, 1/2 liter carafe
N. Fifth, standard bottle
O. Quart, liter, liter carafe
P. Wine Magnum (1.5 liters)

1 Other
2 Pitcher (60 oz)
6 Half gallon
7 Gallon
9 Refused

Type the "Other" size respondent reported.
Press enter twice when you are finished.

Record number of drinks of liquor and select unit on next screen. R's usual liquor size is . Record usual unless another size is reported. Refer to models first; show photo 4 if necessary.

Glasses:

X. 2 oz
A. 4 oz
B. 6 oz
C. 8 oz
D. 10 oz
E. 12 oz
F. 14 oz

Bottles:

G. 50 ML
I. Half a pint
K. Half a fifth
L. Pint, 1/2 liter
N. Fifth, standard bottle
O. Quart, liter
P. Liquor Magnum (1.75 liters)

Shot Glasses:

R. 1.0 oz
S. 1.25 oz

1 Other

9 Refused

Type the "Other" size respondent reported.
Press enter twice when you are finished.

Show Card I-12

65. During the past 30 days, how often did you drink more than usual?

1 Once a week or more
2 Three days in the past 30 days
3 Two days in the past 30 days
4 Once in the past 30 days
9 Refused

Show Cards I-12A and I-12B

66. Now think about all the times you had alcohol to drink during the past 30 days. Out of 10 times when you drank alcohol, how often did you drink with a meal, how often did you drink while snacking, and how often did you have something to drink without eating anything?

Multiply response by 10 and record: 1 = 10, 2 = 20, etc. If respondent says less than one, record as 5 and adjust numbers accordingly. They must add to 100. If respondent refuses to answer, enter 999 instead of 100.

Drank with meals
Drank while snacking
Drank without eating anything

Show Cards I-13A and I-13B

67. Of all the beers that you drank during the past 30 days, how many out of 10 were (***each type of beer)***?

Multiply response by 10 and record: 1 = 10, 2 = 20, etc. If respondent says less than one, record as 5 and adjust numbers accordingly. They must add to 100. If respondent refuses to answer, enter 999 instead of 100.

Show Cards I-13A and I-13B

68. Out of every ten drinks of wine you had during the past 30 days, how many out of 10 were ***(each type of wine)***?

Multiply response by 10 and record: 1 = 10, 2 = 20, etc. If respondent says less than one, record as 5 and adjust numbers accordingly. They must add to 100. If respondent refuses to answer, enter 999 instead of 100.

Show Cards I-13A and I-13B

69. Out of every ten drinks of liquor you had during the past 30 days, how many out of 10 were (***each type of liquor***)?

Multiply response by 10 and record: 1 = 10, 2 = 20, etc. If respondent says less than one, record as 5 and adjust numbers accordingly. They must add to 100. If respondent refuses to answer, enter 999 instead of 100.

Show Card I-13A and I-13B

70. Again, in the past 30 days, thinking of all the major beverage types you had to drink, out of every ten drinks that you had, how many were ***(major beverages as reported in Q43)***?

Multiply response by 10 and record: 1 = 10, 2 = 20, etc. If respondent says less than one, record as 5 and adjust numbers accordingly. They must add to 100. If respondent refuses to answer, enter 999 instead of 100.

71. During this period, did you ever drink enough to get drunk or very high, that is, your speech was slurred or you were unsteady on your feet?

1 Yes
2 No (Skip to CLDH.)
9 Refused (Skip to CLDH.)

Show Card I-14

72. How often did you drink enough to get drunk or very high?

1 5 days or more a week
2 3 to 4 days a week
3 1 to 2 days a week
4 1 to 3 days in the past 30 days
9 Refused

COGNITIVE LIFETIME DRINKING HISTORY
Floating Age Version

Fill out/go over the lifetime calendar.

Continue looking at the calendar as we go through the next series of questions. Thinking about what you were doing during different times of your life - where you were living, whether you were working, and other things - can help you remember your drinking patterns.

1. How old were you when you began to drink on a more or less regular basis? By this we mean having a drink at least once a month for a period of at least six months.

   Enter age.

   If respondent reports that (s)he drank 12 drinks a year but irregularly (for example, a 'holiday drinker'), not drinking at least once a month for a period of at least six months, enter 97. This skips you to the last section, irregular drinkers, question 36.

   99 Refused

   Now I want you to think of times in your life when there were major changes in your drinking pattern, major increases, decreases, or periods when you stopped drinking.

2. How old were you when the (first/next) major change took place?

   Enter age.

   If respondent reports his/her drinking pattern never changed (or did not change again), enter 96. This skips you to question 8.

   99 Refused

3. Why did your drinking change?

   Enter responses from Reasons for Major Change in Drinking Pattern.

4. (After your drinking changed,) did you continue to drink some kind of alcoholic beverage at least once a month?

   1 Yes ***Identifies a drinking phase. Program will cycle through questions 2-7 until all phases have been recorded.***
   2 No ***Identifies beginning of a non-drinking phase. Ask questions 5-7.***
   9 Refused

5. Did you ever start drinking regularly again, that is, at least once a month for a period of at least six months?

   1 Yes
   2 No ***(Skip to Q8.)***
   9 Refused

6. What was the reason for this change?

   Enter responses from Reasons for Major Change in Drinking Pattern.

7. How old were you when you started drinking regularly again?

   Enter age.

   99 Refused

If drinking pattern in last interval (including past 12 months) contradicts drinking pattern reported in the Introduction to the Cognitive Lifetime Drinking History, ask Q7A:

7A. Let's see, I recorded earlier that you drank/didn't drink in the past 12 months. However, we just talked about your recent not drinking/drinking. In the past 12 months, did you have at least one drink a month for a period of at least six months?

1 Yes
2 No
9 Refused

If Respondent refuses to answer Q7A, the information given in the Cognitive Lifetime DrinkingHistory is used to complete the rest of the interview.

Show card F1.

If respondent reported drinking in the past year, ask:

8. Let's see, you already told me about drinking ***(beverages in the past year)***. Did you ever drink other major beverages regularly in your lifetime? By regularly we mean at least once a month for a period of at least six months.

   (Check additional beverages that apply.)

   Beer?

   Wine cooler?

   Wine?

   Liquor?

If respondent did not report drinking within the past 12 months (Question 1 in the Introduction=2), ask:

Which of the major beverage types: beer, wine cooler, wine, and liquor, did you ever drink regularly in your lifetime? By regularly, we mean at least once a month for a period of at least six months.

(Check all that apply.)

Beer?

Wine cooler?

Wine?

Liquor?

If drink size has not been reported, ask:

9. People define a drink of ***(beverage from question 8: beer, wine cooler, wine, liquor)*** in different ways. What size ***(beverage from question 8)*** did you drink when you first started to drink ***(beverage from question 8)*** regularly?

Enter and ask:

10. Did your ***(beverage from question 9)*** size stay the same throughout your lifetime?

1 Yes
2 No
9 Refused

If drink size has already been reported, ask:

11. Your most recent ***(beverage from question 8: beer, wine cooler, wine, liquor)*** size was .... Was this your ***(beverage from question 8)*** size throughout your lifetime?

1 Yes (Skip to Q12.)
2 No
9 Refused

11A. What was your ***(beverage from question 8: beer, wine cooler, wine, liquor)*** size when you first began to drink ***(beverage from question 8)*** regularly?

Enter beverage size.

Questions 12 through 32 constitute the questions asked for each drinking phase. Program will remind respondent of the age phase being assessed. Lifetime events calendar can be referred to as necessary.

Show card F2.

12. From the age when you began to drink regularly until your first major change in drinking pattern, that is, from ages ... to ..., how often did you drink some kind of alcoholic beverage?

1 Once a week or more often *(Skip to Q14.)*
2 Three days a month
3 Two days a month
4 Once a month
9 Refused

For subsequent phases, ask:

From age ... until your next major change at age ..., how often did you drink some kind of alcoholic beverage?

13. On those days when you drank alcohol, how many drinks did you usually have?

Enter number of drinks. ***(Skip to Q24.)***

99 Refused

14. Now we want to know about your drinking patterns (when you first began to drink regularly/beginning age...). We will ask about drinking on weekends and weekdays.

Let's start with Friday. On Fridays during a typical month, how often did you drink - every Friday, three Fridays, two Fridays, one Friday, or hardly ever on Fridays?

4 Every Friday
3 Three Fridays
2 Two Fridays
1 One Friday
0 Hardly ever on Fridays (Skip to Q16.)
9 Refused

15. How many drinks would you usually have on a Friday?

Enter number of drinks.

99 Refused

16. Now think about Saturdays. On Saturdays during a typical month back then, how often did you drink - every Saturday, three Saturdays, two Saturdays, one Saturday, or hardly ever on Saturdays?

    4 Every Saturday
    3 Three Saturdays
    2 Two Saturdays
    1 One Saturday
    0 Hardly ever on Saturdays (Skip to Q18.)
    9 Refused

17. How many drinks would you usually have on a Saturday?

    Enter number of drinks.

    99 Refused

18. On Sundays during a typical month from ages ... to ..., how often did you drink - every Sunday, three Sundays, two Sundays, one Sunday, or hardly ever on Sundays?

    4 Every Sunday
    3 Three Sundays
    2 Two Sundays
    1 One Sunday
    0 Hardly ever on Sundays (Skip to Q20.)
    9 Refused

19. How many drinks would you usually have on a Sunday?

    Enter number of drinks.

    99 Refused

20. Now think about the four weekdays, Monday through Thursday. How often during a typical month did you usually drink on a weekday?

    Show card F3.

1 All four weekdays, every week (16 weekdays)
2 Three weekdays, every week (12 weekdays)
3 Two weekdays, every week (8 weekdays)
4 One weekday, every week (4 weekdays)
5 One to three weekdays a month (1-3 weekdays)
6 Hardly ever on weekdays (0 weekdays)
7 Some other pattern. Try to fit respondent's frequency of weekday drinking during a typical month into codes above, i.e. 16 days a month, 12 days a month, etc. or code 7 and use Control-Note to record unusual frequency pattern reported.
9 Refused

21. How many drinks would you usually have on a weekday?

Enter number of drinks.

99 Refused

If respondent has answered "hardly ever" to drinking on all seven weekdays, the computer will call up Q22 and Q23:

22. About how many days a week, on average, did you drink alcohol from ages .... to ....?

Enter number of days.

99 Refused

23. On those days, how many drinks on average did you usually have?

Enter number of drinks.

99 Refused.

24. From ages ... to ..., were there days when you had more than your usual?

1 Yes
2 No
9 Refused

Show card F4.

25. How often would you drink more than usual?

1 Once a week or more
2 Three days a month
3 Two days a month
4 Once a month
5 7 to 11 days a year
6 3 to 6 days a year
7 Two days a year
8 Once a year or less
9 Refused

26. On a day when you had more than usual, how many drinks in total would you typically have?

Enter number of drinks.

99 Refused

Show card F5.

27. From ages ... to ..., how often did you have enough alcohol to get drunk or very high, that is, your speech was slurred or you were unsteady on your feet?

01 Once a week or more
02 Three days a month
03 Two days a month
04 Once a month
05 7 to 11 days a year
06 3 to 6 days a year
07 Two days a year
08 Once a year or less
09 Never in this time period
10 Never in my entire life
99 Refused

Show cards F6A & F6B.

28. From ages ... to ..., out of 10 times when you drank alcohol, how often did you drink with meals or snacks and how often without?

Multiply response by 10 and record 1=10, 2=20, etc. If respondent says less than one, record as 5 and adjust numbers accordingly. They must add to 100. If respondent refuses to answer, enter 999 instead of 100.

Drank with meals or snacks
Drank without eating anything

For subsequent phases, ask:

How often did you drink with meals or snacks, and how often without?

Show card F7.

29. Out of every ten drinks that you had from ages ... to ..., how many were ***(beverages from question 8)***?

Respondent's lifetime list of major beverages will be generated. Enter the estimated percentage of drinks of each type for the phase under assessment. If a beverage was drunk during the lifetime, but not during a given phase, enter a zero. If respondent says less than one, record as 5 and adjust numbers accordingly. They must add to 100. If respondent refuses to answer, enter 999 instead of 100.

If respondent reported variable drink size during lifetime, for interval 2 and subsequent intervals ask:

30. Your previous ***(beverage(s) from question 29 greater than zero)*** size was....Was this your drink size from ages ... to ...?

1 Yes (Skip to Q32.)
2 No
9 Refused

31. What was your ***(beverage(s) from question 29 greater than zero)*** size during the period beginning age ...?

Enter beverage size.

32. Did your ***(beverage(s) from question 29 greater than zero)*** size stay the same the rest of your life?

1 Yes
2 No
9 Refused

Repeat Questions 12 through 32 for each phase reported.

Show cards F8A & F8B.

33. Of all the beers that you drank during your lifetime, how many out of 10 were ***(each type of beer)***?

34. Out of every ten drinks of wine you had during your lifetime, how many out of 10 were ***(each type of wine)***?

35. Out of every ten drinks of liquor that you had during your lifetime, how many out of 10 were ***(each type of liquor)***?

Questions for Irregular (Holiday) Drinkers

36. During the period of your life when you drank the most, how many times a year did you drink?

Enter number of times.

99 Refused

37. When you drank during these times, how many drinks did you usually have?

Enter number of drinks.

99 Refused

38. Were there days when you had more than your usual?

1 Yes
2 No (Skip to Q41.)
9 Refused

39. How often would you have more than usual?

Enter number of times/year.

99 Refused

40. On a day when you had more than usual, how many drinks would you typically have?

Enter number of drinks.

99 Refused

41. During this period, did you ever drink enough to get drunk or very high, that is, your speech was slurred or you were unsteady on your feet?

1 Yes
2 No (Skip to Q43.)
9 Refused

42. How often did you drink enough to get drunk or very high?

Enter number of times/year.

99 Refused

43. How old were you during this pattern of drinking?

Enter ages: .... to .....

99 Refused

END OF COGNITIVE LIFETIME DRINKING HISTORY.

ADVISEMENT

1. Has your doctor, nurse, or other health care provider, ever recommended any of the following?
   - ☐ Drink moderate amount of alcohol (q.3)
   - ☐ Drink less (q.3)
   - ☐ Drink more (q.3)
   - ☐ Drink less of some certain beverage(s) and more of others (q.3)
   - ☐ Don't drink at all (q.3)
   - ☐ Don't change drinking habits (q.5)
   - ☐ Never discussed with doctor or other health care provider
   - ☐ *Don't Know (q.3)*
   - ☐ *Refused*

3. Were you advised to drink more or less of particular types of alcoholic beverages?
   ☐ Yes (q.4) ☐ No (next section)
   ☐ *Don't Know (next section)* ☐ *Refused (next section)*

4. Which beverages were you advised to drink less of or more of?
   (check as many as apply)

	Drink less	Drink more	Drink the same
Beer	☐	☐	☐
White Wine	☐	☐	☐
Red Wine	☐	☐	☐
Liquor	☐	☐	☐
Other_____	☐	☐	☐

REASONS FOR MAJOR CHANGE IN DRINKING PATTERN

1. Significant/family relationships
    1. Got married or started a significant relationship other than marriage
    2. Significant/family relationship changed
    3. Separated, divorced, annulment, ended a significant relationship other than marriage
    4. Widowed; death of significant person

2. Respondent's health status
    1. Heart disease
    2. Lung cancer
    3. Any other illness or injury, including surgery
    4. Health improved
    5. Pregnancy
    6. Alcohol/medication interactions
    7. Physician advice
    8. General health concerns about alcohol, including weight control
    9. Mental health status improved or worsened
    10. Other

3. Work
    1. Started a new job
    2. Changed job or career
    3. Lost job/unemployed
    4. Experienced pressure at job
    5. Retired
    6. Other

4. Armed services

5. School
    1. Started school
    2. Left school
    3. Experienced pressure at school

6. Changed residence

7. Legal reasons/Jail

8. Financial reasons

9. External pressure
    1. Peers
    2. Family
    3. Comments from children
    4. Employer
    5. Minister, priest, rabbi
    6. Other

10. Drug abuse
    1. Started using drugs or increased use
    2. Stopped using drugs or decreased use

11. Alcohol and/or drug related treatment
    1. Alcohol treatment: started, changed, stopped
    2. Drug dependency treatment: started, changed, stopped
    3. Alcohol and drug dependency treatment: started, changed, stopped

12. Religious reasons

13. Social reasons
    1. Sports
    2. More family responsibilities
    3. Fewer family responsibilities
    4. Socializing pattern changed

14. Alcohol/drinking related reasons
    1. Legal age to drink changed pattern of drinking
    2. Negative drinking or drug experience
    3. Fear of consequences of drinking
    4. Attitude changed
    5. Taste changed
    6. Effect of alcohol changed
    7. Cost of alcohol changed pattern of drinking
    8. Self-help program
    9. Other

15. Other

16. No specific reason

99. Refused

We will now be asking questions related to your smoking and other forms of tobacco exposure.

SMK1 103. During your entire life, have you smoked at least 100 **cigarettes**, that is about 5 packs of cigarettes?
☐ Yes ☐ No (q.111) ☐ *Don't Know*
☐ *Refused (q.111)*

104. How old were you when you first started smoking cigarettes regularly?

Age: _____

105. Do you smoke cigarettes now?
☐ Yes (q.111) ☐ No ☐ *Don't Know*
☐ *Refused (q.111)*

106. How old were you when you quit smoking regularly?

Age: _____

SMK9A 111. Have you smoked a **pipe with tobacco** at least 20 times in your life?
☐ Yes ☐ No (q.115) ☐ *Don't Know*
☐ *Refused (q.115)*

111a. Have you smoked a pipe regularly for at least 6 months during your life?
☐ Yes ☐ No (q.115) ☐ *Don't Know*
☐ *Refused (q.115)*

111b. When you smoked a pipe of tobacco, did you inhale the smoke into your lungs?
☐ Yes, inhaled ☐ No ☐ *Don't Know*
☐ *Refused*

112. How old were you when you first started smoking a pipe regularly?
Age: _____

113. Do you smoke a pipe now?
☐ Yes (q.115) ☐ No ☐ *Don't Know*
☐ *Refused (q.13)*

114. How old were you when you quit smoking a pipe regularly?

Age: _____ (then to q.115)

SMK14A 115. Have you smoked at least 20 **cigars** in your life?
☐ Yes ☐ No (q.122) ☐ *Don't Know*

☐ *Refused (q.122)*

116. Have you smoked cigars regularly for at least 6 months during your life?
☐ Yes ☐ No (q.122) ☐ *Don't Know*
☐ *Refused (q.122)*

117. When smoking cigars, did you inhale the smoke into your lungs?
☐ Yes, inhaled ☐ No ☐ *Don't Know*
☐ *Refused*

118. How old were you when you first started smoking cigars regularly?

Age: _____

119. Do you smoke cigars now?
☐ Yes (q.122) ☐ No ☐ *Don't Know*
☐ *Refused (q.122)*

120. How old were you when you quit smoking cigars regularly?

Age: _____ (then to q.122)

SMK19A 122. Have you used **chewing tobacco** at least 20 times in your life?
☐ Yes ☐ No (q.128) ☐ *Don't Know*
☐ *Refused (q.128)*

123. Have you used chewing tobacco regularly for at least 6 months during your life?
☐ Yes ☐ No (q.128) ☐ *Don't Know*
☐ *Refused (q.128)*

124. How old were you when you first started chewing regularly?

Age: _____

125. Do you chew tobacco now?
☐ Yes (q.128) ☐ No ☐ *Don't Know*
☐ *Refused (q.128)*

126. How old were you when you quit chewing tobacco regularly?

Age: _____ (then to q.128)

SMK24A 128. Have you used **snuff**, such as Skoal, Skoal Bandits, or Copenhagen, at least 20 times in your life?

☐ Yes ☐ No (q.135) ☐ *Don't Know*
☐ *Refused (q.135)*

129. Have you used snuff regularly for at least 6 months during your life?
☐ Yes ☐ No (q.135) ☐ *Don't Know*
☐ *Refused (q.135)*

130. How old were you when you first started using snuff regularly?

Age: _____

131. Do you use snuff now?
☐ Yes (q.135) ☐ No ☐ *Don't Know*
☐ *Refused (q.135)*

132. How old were you when you quit using snuff regularly?

Age: _____ (then to q.135)

SMK29A 135. Have you smoked **marijuana, pot, or hashish** at least 20 times in your lifetime?
☐ Yes ☐ No (q.141) ☐ *Don't Know*
☐ *Refused (q.141)*

136. How old were you when you first smoked marijuana, pot, or hashish?

Age: _____

137. Do you smoke marijuana, pot, or hashish now?
☐ Yes (q.141) ☐ No ☐ *Don't Know*
☐ *Refused (q.141)*

138. How old were you when you quit smoking marijuana or hashish?

Age: _____ (then to q.141)

141. Have you ever **freebased or smoked crack** at least 20 times in your life?
☐ Yes ☐ No (next section) ☐ *Don't Know*
☐ *Refused (next section)*

142. How old were you when you first freebased or smoked crack?

Age: _____

143. Do you freebase or smoke crack now?
☐ Yes (next section) ☐ No ☐ *Don't Know*
☐ *Refused (next section)*

144. How old were you when you quit freebasing/smoking crack?

Age: _____ (to next section)

MEN ONLY

These next two questions are about when you were first born.

73. What was your birth weight? *(Show Card 26)*
- ☐ < 5 lbs.
- ☐ 5 to 5½ lbs.
- ☐ More than 5½ to 7 lbs.
- ☐ More than 7 to 8½ lbs.
- ☐ More than 8½ to 10 lbs.
- ☐ More than 10 lbs.
- ☐ *Refused*
- ☐ *Don't Know exactly, but*
  - ☐ *Above average*
  - ☐ *Below Average*
  - ☐ *Average*
  - ☐ *Don't Know*

73a. Were you breast fed as an infant?
☐ Yes ☐ No ☐ *Don't Know* ☐ *Refused*

74. We would now like to know what your weight was one year ago? _____

Now, a few questions about physical changes that may have occurred during your adult life.

74a. Please select from these 9 silhouettes the one that best represents the shape of your body at about 14 years of age. *(Show Card 27)*

75a. Has your hairline receded?
☐ Yes ☐ No ☐ *Don't Know* ☐ *Refused*

75b. (Only if yes to 75a.) At what age did your hairline begin to recede? (approximately) _____ yrs. old

76a. Have you lost hair from the crown of your head?
☐ Yes ☐ No ☐ *Don't Know* ☐ *Refused*

76b. (Only if yes to 76a.) At what age did you begin to lose hair from the crown of your head? (approximately) _____ yrs. old

77a. Have you ever had a vasectomy?
☐ Yes ☐ No ☐ *Don't Know* ☐ *Refused*

77b. (If yes) At what age? _____ yrs. old

WOMEN ONLY

These next two questions are about when you were first born.

78. What was your birth weight? *(Show Card 26)*

☐ < 5 lbs.
☐ 5 to 5½ lbs.
☐ More than 5½ to 7 lbs.
☐ More than 7 to 8½ lbs.
☐ More than 8½ to 10 lbs.
☐ More than 10 lbs.
☐ *Refused*
☐ *Don't Know exactly, but*
☐ *Above Average*
☐ *Below Average*
☐ *Average*
☐ *Don't Know*

78a. How old was your mother when you were born?

1) less than 18
2) 18 to 20
3) 21 to 25
4) 26 to 30
5) 31 to 35
6) more than 35
8) Don't know
9) Refused

78b. Among your brothers and sisters, where were you in the order of birth?

1) First child (Oldest)
2) Second child
3) Third child
4) Fourth child or Higher
8) Don 't know
9) Refused

78c. Were you breast fed as an infant?

☐ Yes ☐ No ☐ *Don't Know* ☐ *Refused*

79. We would now like to know what your weight was one year ago? _____

These next few questions are about your development as a woman.

80. At what age did you have your first menstrual period? _____ yrs. old (Give your best guess if not exactly sure.)

80b. Please select from these 9 silhouettes the one that best represents your body shape at about the time of your first period. *(Show Card 27)*

81. In the first year after your period began, were your cycles regular or irregular compared to how they were for the rest of your life (by regular, I mean that you could predict the week of your next period)?
☐ Regular
☐ Irregular
☐ Always irregular during menstruating years
☐ *Don't Know*
☐ *Refused*

82. Many women experience a growth spurt between 7 and 18 years of age. We would like to know at approximately what age this happened for you. We do not want to know your exact age, but whether your growth spurt happened before or after you began menstruating.

☐ Before menarche
☐ Same time as menarche
☐ After menarche
☐ Never happened to me
☐ *Don't Know*
☐ *Refused*

83. Have your menstrual periods ceased permanently?
☐ Yes, go to 85
☐ No, go to 84
☐ Not sure, explain _____ then go to question 86

84. When was the first day of your last menstrual period? (Give day and month if you are still menstruating.) *(Show Card 28)*
Month: __ Day: __ Year: 19__
(skip to 87b.)

85. At what age was your last period? _____ yrs. old

86. For what reason did your menstrual periods cease?
☐ Naturally (menopause) to 87b
☐ Surgery (hysterectomy), answer question 87
☐ Radiation or Chemotherapy to 87b
☐ Other (specify) to 87b
☐ *Don't Know to 87b*
☐ *Refused to 87b*

87. Were your ovaries removed (probes: After surgery did you have "sudden menopause" or take hormone replacement therapy? Were you told it was a total or partial hysterectomy?)

☐ No, only uterus removed

☐ Yes, both ovaries removed

☐ Yes, only one ovary removed

☐ *Don't Know*

☐ *Refused*

PREGNANCIES AND CHILDBIRTH (WOMEN ONLY)
This section of questions will focus on your pregnancies and childbirth.

92a. We would now like some information about any pregnancies you may have had, including live births, miscarriages, still births, tubal pregnancies or elective or therapeutic abortions. How many pregnancies have you had? _____ (If zero, next question. If one or more, skip to 93a. If 88 = D/K or 99 = R skip out of section.)

92b. (If zero pregnancies) Were you unable to become pregnant or did you not become pregnant by choice?
☐ Unable ☐ Choice ☐ *Don't Know* ☐ *Refused*
(Skip to next section)

First pregnancy only, if more than one pregnancy: first, I will be asking a few questions about each pregnancy, beginning with your first pregnancy.

93a. How old were you when your first pregnancy ended? _____

93b. What was the outcome of your first pregnancy?
(Show Card 29)
o Full term, live birth (ask 93d to 93l)
o Pre-term, live birth (ask 93d to 93l)
o Miscarriage (ask 93c, then skip to next pregnancy or out)
o Still Born (ask 93c to 93f, then to next pregnancy or out)
o Early termination by elective or therapeutic abortion (ask 93c, then skip to next pregnancy or out)
o Born Alive, died after birth (ask 93c to 93f, then skip to next pregnancy or out)
o Tubal pregnancy or Ectopic pregnancy (ask 93c, then skip to next pregnancy or out)
o Other (Specify): _____

☐ *Don't Know (88)* ☐ *Refused (99)*

93c. How long did you carry this pregnancy? (How long were you pregnant? How many weeks or months long was this pregnancy?) _____weeks or _____months
☐ *Don't Know* ☐ *Refused*

93d. On a scale of 1 to 5, how much nausea did you experience during your first pregnancy? One is very little nausea, five is extreme nausea.
☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5
☐ zero (no nausea, skip 93e)
☐ *Don't Know*
☐ *Refused*

93e. How long during the pregnancy did you experience nausea? The first trimester, into the second trimester, or into the third trimester?
☐ First ☐ Second ☐ Third ☐ *Don't Know*
☐ *Refused*

93f. About how much weight did you gain during this pregnancy?
_____ pounds ☐ *Don't Know* ☐ *Lost weight*

93g. Did you breast feed this child? ☐ Yes (93i) ☐ No (93h)
☐ *Don't Know (skip to next pregnancy or out)*
☐ *Refused*

93h. Did you decide to bottle feed from the beginning or did you have difficulty with breast feeding? (skip to next pregnancy or out)
☐ Tried ☐ Bottle ☐ *Don't Know* ☐ *Refused*

93i. How many months did you breast feed the child? (allow for decimals to record by week) _____
☐ *Don't Know* ☐ *Refused*

93j. How old was your infant when you started giving him/her anything other than breast milk (such as formula, milk, cereal, other baby foods)?
_____ Weeks old _____Months old
☐ *Don't Know* ☐ *Refused*

93k. Did you stop breast feeding because you could not produce enough milk?
☐ Yes ☐ No ☐ *Don't Know* ☐ *Refused*

93l.. After your child was born, how long was it before you had a menstrual period? (*Show Card 29A*)
1) 1 Month or less
2) 2 – 4 Months
3) 4-6 Months
4) 6 – 10 Months
5) 10 – 12 Months
6) More Than 12 Month
8) DON'T KNOW
9) REFUSED

(Continue intervals with 2nd through 10th pregnancies in same format.)

If more than one pregnancy: Now a few questions about all of your pregnancies considered together.

95. *(If one pregnancy:* Was your pregnancy a twin or multiple birth?)
Were any of your pregnancies twin or multiple births?
☐ No (all singleton births)
☐ Twins
☐ Triplets or more
☐ *Don't Know*
☐ *Refused*

95a. Did you have regular prenatal visits or see a health care provider about once a month or more often during your pregnancy(s)?
☐ Yes: If more than one, for how many of your pregnancies (did you have monthly prenatal visits)? _____
☐ No ☐ *Don't Know* ☐ *Refused*

95b. Did you experience bleeding gums (that occurred while not brushing or flossing your teeth) tumor of the gums or periodontal-gingival disease during your pregnancy(s)?
☐ Yes: If more than one, for how many of your pregnancies (did you have bleeding gums)? _____
☐ No ☐ *Don't Know* ☐ *Refused*

95c. Were you treated for high blood pressure during your pregnancy(s) (*Prompt:* did you begin treatment for high blood pressure during your pregnancy(s), including treatment by medications or dietary changes)?
☐ Yes: *If more than one,* for how many of your pregnancies (were you treated for high blood pressure)? _____
☐ No (skip 95e) ☐ *Don't Know (skip 95e)*
☐ *Refused*

95d. Was this (the high blood pressure) short term or treated after your pregnancy(s) too?
☐ Short term (Treated only during pregnancy(s))
☐ Sustained after pregnancy (for one or more of your pregnancies)
☐ *Don't Know*

95e. Were you diagnosed with preeclampsia, eclampsia, or toxemia (*Prompt:* That is high blood pressure during or after the 4th month of pregnancy, swelling, and protein in your urine, toxemia is having seizures as a complication)?
☐ Yes: If more than one, for how many of your pregnancies (did you have preeclampsia, eclampsia or toxemia)? _____
☐ No ☐ *Don't Know* ☐ *Refused*

95f. Were you diagnosed with gestational diabetes or hyperglycemia during your pregnancy(s)? (*Prompt:* That is high sugar in your blood or sugar in your urine)

☐ Yes: If more than one, for how many of your pregnancies (did you have gestational diabetes)? _____
☐ No (skip out) ☐ *Don't Know (skip out)*
☐ *Refused*

95g. Did the diabetes go away after delivery?
☐ Yes ☐ No ☐ *Don't Know* ☐ *Refused*

95h. Did your breast size increase, decrease or remain the same during your pregnancy(s)?

☐ Increased (For at least one pregnancy)
☐ Decreased
☐ Remained the same
☐ *Don't Know*
☐ *Refused*

Next, I want to ask you some questions about you sleeping habits. Think about a usual month in the 12 months that ended one year ago, that is, the period from jul 1995 to jul 1996.

1. In a usual 24-hour period in that year, about how much did you sleep? (*Show Card 28A*)

1. Less than 4 hours
2. 4 – 5 hours
3. 6 – 7 hours
4. 8 hours
5. 9 – 10 hours
6. More Than 10 hours
8. DON'T KNOW
9. REFUSED

2. In a usual month in that year, how often did you have trouble falling asleep? (*Show Card 28B*)

1. Not at all
2. 1 – 3 days
3. 4 – 7 days
4. 8 – 14 days
5. 15 – 21 days
7. 22 – 31 days
8. DON'T KNOW
9. REFUSED

3. In a usual month in that year, how often did you wake up several times each night? (*Show Card 28B)*

1. Not at all
2. 1 – 3 days
3. 4 – 7 days
4. 8 – 14 days
5. 15 – 21 days
6. 22 – 31 days
8. DON'T KNOW
9. REFUSED

4. In a usual month in that year, how often did you have trouble staying asleep (including waking up far too early? (*Show Card 28B)*

1. Not at all
2. 1 – 3 days
3. 4 – 7 days
4. 8 – 14 days
5. 15 – 21 days
6. 22 – 31 days
8. DON'T KNOW
9. REFUSED

5. In usual month in that year, how often did you wake up after your usual amount of sleep feeling tired and worn out? (*Show Card 28B*)

1. Not at all
2. 1 – 3 days
3. 4 – 7 days
4. 8 – 14 days
5. 15 – 21 days
6. 22 – 31 days
8. DON'T KNOW
9. REFUSED

NSAIDS SECTION:

These next few questions are about your use of pain medicines, especially the use of aspirin, ibuprofen, or aspirin-free products, during the period 12-24 months ago.

1. On average, how frequently do you take **ibuprofen**, such as Advil, Nuprin, Motrin, Aleve? *(Show Card 30A)*
   ☐ < 1 day/mo ☐ 1-3 days/mo ☐ 1-2 days/wk
   ☐ 3-4 days/wk ☐ 5-6 days/wk ☐ Daily
   ☐ Never ☐ *Don't Know* ☐ *Refused*

2. On average, how many tablets (of ibuprofen) do you take per day? *(Show Card 30B)*
   ☐ ¼ Tablet ☐ ½ Tablet ☐ 1 Tablet ☐ 2 Tablets
   ☐ 3-4 Tablets ☐ 5-6 Tablets ☐ 7 or more Tablets
   ☐ Not Applicable ☐ *Don't Know* ☐ *Refused*

3. On average, how frequently do you take **acetaminophen** the brand names are Tylenol, Panadol, Excedrin P.M. or aspirin-free pain pills? *(Show Card 30A)*
   ☐ < 1 day/mo ☐ 1-3 days/mo ☐ 1-2 days/wk
   ☐ 3-4 days/wk ☐ 5-6 days/wk ☐ Daily
   ☐ Never ☐ *Don't Know* ☐ *Refused*

4. On average, how many tablets (of acetaminophen) do you take per day? *(Show Card 30B)*
   ☐ ¼ Tablet ☐ ½ Tablet ☐ 1 Tablet ☐ 2 Tablets
   ☐ 3-4 Tablets ☐ 5-6 Tablets ☐ 7 or more Tablets
   ☐ Not Applicable ☐ *Don't Know* ☐ *Refused*

5. On average, how frequently do you take **aspirin**? *(Show Card 30A)*
   ☐ < 1 day/mo ☐ 1-3 days/mo ☐ 1-2 days/wk
   ☐ 3-4 days/wk ☐ 5-6 days/wk ☐ Daily
   ☐ Never ☐ *Don't Know* ☐ *Refused*

6. On an average, how many aspirin tablets do you take <u>on days when you take aspirin</u>? (If you take baby aspirin - one baby aspirin tablet equals one-quarter of a regular aspirin tablet. Please include regular Anacin, Bufferin, Midol, Alka Seltzer, Extra Strength Excedrin etc., but not aspirin-free products or Tylenol.) *(Show Card 30B)*
   ☐ ¼ Tablet ☐ ½ Tablet ☐ 1 Tablet ☐ 2 Tablets
   ☐ 3-4 Tablets ☐ 5-6 Tablets ☐ 7 or more Tablets
   ☐ Not Applicable ☐ *Don't Know* ☐ *Refused*

Next a few questions about your teeth and oral health.

1. How many of your adult (permanent) teeth have you lost (had pulled or extracted?)____ (If 0 skip 2)
(Not including wisdom teeth/third molars, but include other molars or premolars or incisors pulled for braces/orthodontia.)
☐ *Don't Know* ☐ *Refused*

2. In the last 2 years, how many teeth have you lost (had pulled or extracted)?_____ ☐ *Dont Know* ☐ *Refused*

3. In the last 5 years, how many times have you been to the dentist?
☐ Never ☐ 1-3 times ☐ more than 3 times
☐ *Don't Know* ☐ *Refused*

4. Have you ever been told that your have periodontal disease? (PROMPTS: Sometimes called pyorrhea, gum disease which includes loss of the bone that supports the teeth.)
☐ Yes ☐ No ☐ *Don't Know* ☐ *Refused*

4a. If yes/not certain to above:
In the last 2 years, have you had periodontal surgery (surgery on your gums not including root canals)?
☐ Yes ☐ No ☐ *Don't Know* ☐ *Refused*

Lifetime History Questions

This is a series of questions about your lifestyle at certain stages of your life, most of these topics have been covered earlier in the questionnaire, however we want to get an idea of how these factors "fit" together. (To be asked for time periods of 20 years old and younger, 21-30, 31-40, 41-50, 51-60, 61-70, 71-present age.)

(Do not ask 1-4 for 20 years old and younger.)

1. How many close friends did you have, that is people that you felt at ease with, could talk to about private matters, or could call on for help? _____

2. During an average month, how many times did you see some of your close friends or relatives? *(Show Card 31)*
   1. Nearly daily
   2. About once a week
   3. About once a month
   4. or less often
   8. DON'T KNOW
   9. REFUSED

3. If you needed to talk to someone about something very personal, was there someone you could confide in or count on to listen?
   ☐ Yes ☐ No ☐ DON'T KNOW ☐ REFUSED

4. If you needed help, that is if your car broke down or if you needed a ride somewhere, was there someone you could call on to help you out?
   ☐ Yes ☐ No ☐ DON'T KNOW ☐ REFUSED

Now, a question about weight, (during this age period).

(For **20 years old and younger**, replace text with **at age 18.**)

5. What was your approximate weight (not during pregnancy or following an illness)? _____ lbs.

(Only if reported smoking > 100 cigarettes during lifetime assessment – Yes to 103)
Now a few questions about smoking...

1. Between the ages of [ ] and [ ], did you smoke cigarettes?
   ☐ Yes ☐ No (to q.10) ☐ *Don't Know* ☐ *Refused*

2. When you smoked, how many cigarettes on average did you smoke per day? _____ (record number)

4. Were the cigarettes filtered or non-filtered?
   ☐ Filtered ☐ Non-filtered ☐ Both
   ☐ *Don't Know* ☐ *Refused*

5. Were the cigarettes menthol or non-menthol?
   ☐ Menthol ☐ Non-menthol ☐ Both

☐ *Don't Know* ☐ *Refused*

6. Between the ages of [ ] and [ ], did you quit smoking for six months or longer?
☐ Yes ☐ No (next section)
☐ *Don't Know (next section)* ☐ *Refused*

7. Adding together any times you were not smoking for more than 24 hours, how long were you not smoking cigarettes during the time when you were [ ] to [ ] years old?
_____ YEARS _____ MONTHS

(Only if reported smoking pipe <u>regularly</u> during lifetime assessment – Yes to 111a)

10. Between the ages of [ ] and [ ], did you smoke a pipe daily, less than daily, or not at all?
☐ Daily (skip to 12)
☐ Less than daily
☐ Not at all (skip to next section)

11. In a typical month, on how many days would you smoke a pipe?
_____ (record number)
☐ *Don't Know* ☐ *Refused*

12. On the days you did smoke a pipe, how many bowls (pipe full) of tobacco did you smoke on an average day? _____ (record number)
☐ *Don't Know* ☐ *Refused*

13. Between the ages of [ ] and [ ], did you quit smoking a pipe for six months or longer?
☐ Yes ☐ No (next section)
☐ *Don't Know (next section)* ☐ *Refused*

15. Adding together the times you were not smoking for more than 24 hours, how long were you not smoking a pipe during the time when you were [ ] to [ ] years old?
_____ YEARS _____ MONTHS

(Only if reported cigar smoking <u>regularly</u> during lifetime assessment – Yes to 116)

17. Between the ages of [ ] and [ ], did you smoke a cigar every day, some days, or not at all?
☐ Daily (skip to 19)
☐ Less than daily
☐ Not at all (skip to next section)

18. In a typical month, on how many days would you smoke a cigar?
_____ (record number) ☐ *Don't Know* ☐ *Refused*

19. On the days you did smoke a cigar, how many cigars did you smoke on an average day? _____ (record number)
☐ *Don't Know* ☐ *Refused*

20. Between the ages of [ ] and [ ], did you quit smoking a cigar for six months or longer?
☐ Yes ☐ No (next section)
☐ *Don't Know (next section)* ☐ *Refused*

22. Adding together any times you were not smoking for more than 24 hours, how long long were you not smoking cigars? ___ (record months or years)

(Only if reported smoking marijuana during lifetime assessment – Yes to 135)

30. Between the ages of [ ] and [ ], did you smoke marijuana, pot, or hash every day, some days, or not at all?
☐ Every day (skip to 32)
☐ Some days
☐ Not at all (skip to next section)

31. In a typical month, on how many days would you smoke marijuana or pot? _____ (record number)
☐ *Don't Know (to 32)* ☐ *Refused* *(If zero, skip 33, 35, 38)*

32. In a typical month, on how many days would you smoke hashish? _____ (record number)
☐ *Don't Know (to 33)* ☐ *Refused (if zero, skip 34, 37, 38)*

33. During a typical week or month, about how much marijuana or pot would you smoke? (Measured in number of ounces per week or month or by number of joints per week or month.) *(If R reports once every two months, enter 0.50; If every three months, enter 0.33; If every four months, enter 0.25)*
_____ ☐ Joints per ☐ Week
☐ Ounces ☐ Month
☐ *Don't Know (q.35 if no hash)* ☐ *Refused*

34. During a typical week or month, about how much hashish would you smoke? (Measured in number of ounces per week or month or by number of joints per week or month.) *(If R reports once every two months, enter 0.50; If every three months, enter 0.33; If every four months, enter 0.25)*
_____ ☐ Joints per ☐ Week
☐ Ounces ☐ Month
☐ *Don't Know (q.37 if no pot)* ☐ *Refused*

35. Between the ages of [ ] and [ ], did you quit smoking marijuana for six months or longer?
☐ Yes ☐ No (next section)
☐ *Don't Know (next section)* ☐ *Refused*

36. Adding together any times you were not smoking for more than 24 hours, how long were you not smoking marijuana during the time when you were [ ] to [ ] years old?
_____ YEARS _____ MONTHS (next section)

37. Between the ages of [ ] and [ ], did you quit smoking hashish for six months or longer?
☐ Yes ☐ No (next section)
☐ *Don't Know (next section)* ☐ *Refused*

38. Adding together any times you were not smoking for more than 24 hours, how long were you not smoking hashish when you were [ ] to [ ] years old? _____ YEARS _____ MONTHS

These next few questions are about whether or not you were exposed to other people's smoking. (FIRST TIME ONLY: Please think about people with whom you lived or visited who smoked cigarettes, cigars and/or pipes while you were present.)

P1. [ ] to [ ] years old, how many people living with you smoked, and for how many years did you live with them? Remember to include people who smoked cigarettes, cigars and/or pipes (*Prompt:* approximate/average.) (If zero, skip next question.) _____ (#1-20) Smokers
(88 = Don't Know 99 = Refused)
___ person(s) for ___ years (repeat if less than 10)

P3. *(Show Card 32A and 32B)* [ ] to [ ] years old, in a typical month, how often would you go to a restaurant or bar where there was a lot of smoke?
Would you say: ☐ More than once a week
☐ Between once a month and once a week
☐ Less than once a month
☐ Not at all

P4. *(Show Card 32A and 32B)* In a typical month, how often would you attend other social gatherings, such as dances, parties, or gatherings at friends' homes in which smokers were present?
Would you say: ☐ More than once a week
☐ Between once a month and once a week
☐ Less than once a month
☐ Not at all

P5. *(Show Card 32A and 32B)* In a typical month, how often were you at other settings not previously mentioned, such as club meetings, beauty or barber shops, indoor sporting events, bowling alley or bingo halls, in which a lot of smoking was going on?
Would you say: ☐ More than once a week
☐ Between once a month and once a week
☐ Less than once a month
☐ Not at all

P6. Think about any job or jobs that you may have had during this time. Did you have co-workers who smoked cigarettes near you so that you frequently breathed their smoke?
☐ Yes ☐ No ☐ Don't Know ☐ Refused

P7. How many hours in a week were you exposed to this smoke?
1. Less than one hour
2. Two to Seven hours

3. Eight to Fourteen hours
4. Fifteen to Twenty hours
5. Twenty One to Twenty Seven hours
6. Twenty Eight to Thirty Five hours
7. Over Thirty Five hours
8. Don't Know
9. Refused

P8. How many years were you exposed to this smoke during the time?

1. Less than One year
2. Two to Four years
3. Five to Seven years
4. Eight to Ten years
8. Don't Know
9. Refused

Next, a few questions about pain medicines that you may have used.

1. Did you take aspirin between the ages of [ ] and [ ]?
☐ Yes ☐ No ☐ Don't Know ☐ Refused

2. How frequently did you take aspirin and for how long? *(Show Card 33)*
☐ < 1 day/mo ☐ 1-3 days/mo ☐ 1-2 days/wk
☐ 3-4 days/wk ☐ 5-6 days/wk ☐ Daily
☐ Not Applicable ☐ *Don't Know* ☐ *Refused*

for ___ days, ___ months, ___ years

(21-21b: every interval until menopause)

21. Were you taking birth control pills between the ages of [ ] and [ ]?
☐ Yes ☐ No (q.22) ☐ *Don't Know (q.22)*
☐ *Refused (q.22)*

21a. What was the name or type of your birth control pill? *(Show birth control pills chart)*
_____ ☐ *Don't Know*
☐ *Refused (q.22)*

21b. How many years or months did you take the birth control pill between the ages of [ ] and [ ]?
_____ years _____ months ☐ *Don't Know* ☐ *Refused*

These are a few questions about your employment...

1. Were you out of work for <u>more than six months</u> during the ten years, between the ages of [ ] and [ ]? (AT LESS THAN 20 YEARS OF AGE: From the time you first began working regularly.)
☐ Yes (next question)

☐ No (next section)
☐ *Don't Know (next question)*
☐ *Refused (next section)*

2. How many times were you out of work for more than six months during those ten years? (AT LESS THAN 20 YEARS OF AGE: From the time you first began working regularly.)
☐ Record number (next question)
☐ *Don't Know (next question)*
☐ *Refused (next section)*

(**ADD LOGIC SO THAT QUESTION 3 IS ASKED FOR EACH TIME.)

3. Why were you out of work (the first/second/third/etc. time)? There may be more than one reason listed, so please choose all that apply. *(Show Card 34)*
☐ I was retired during this time (PROBE: Forced or not?)
☐ I was disabled or on sick leave time
☐ I was caring for a child or infant during this time
☐ I was caring for an elderly person during this time
☐ I was in school during this time
☐ I was in jail or prison during this time
☐ I was <u>not</u> actively seeking employment during this time (HOMEMAKER)
☐ I was laid off, dismissed, or looking for employment during this time
☐ *Don't Know*
☐ *Refused*

Begins age 21-30

1) Did you do a breast self-exam?
   1. YES
   2. NO
   8. DON'T KNOW
   9. REFUSED

2) How often did you do a breast self-exam?
   1. Almost every month or menstrual cycle
   2. Sporadically throughout the year
   8. DON'T KNOW
   9. REFUSED

3) Did you have a mammogram done?
   1. YES
   2. NO
   8. DON'T KNOW
   9. REFUSED

4) How many mammograms did you have done during this time period?
   1. One
   2. Two
   3. Three or Four
   4. Five or More
   8. DON'T KNOW
   9. REFUSED

5) During this time period, why did you have the mammogram(s) done?
   1. I had symptoms (for example, felt a lump, breast changes, discharge)
   2. It was a routine test for screening, I did not have symptoms.
   3. I had mammograms both for symptoms and for routine screening.
   8. DON'T KNOW
   9. REFUSED

6) Why did you not have a mammogram? (Please indicate all that apply.)
   I didn't feel like I needed a mammogram
   My doctor didn't recommend or encourage me to get a mammogram
   I did not know about getting a mammogram.
   I was afraid of getting cancer.
   The cost was too high.
   There was no place to go to get a mammogram.
   Other
   DON'T KNOW
   REFUSED
   NONE/NO MORE/NEXT QUESTION

7) During this time period did you have a breast biopsy done?
   1. Yes
   2. No
   8. DON'T KNOW
   9. Refused

8) How many times did you have a biopsy done in this time period (do not include removal of fluid form a fluid-filled cyst)?
   1. 1 Time
   2. 2 Times
   3. More than 2 times
   8. DON'T KNOW
   9. REFUSED

9) How old were you when the first biopsy was done?
   888 DON'T KNOW
   999 REFUSED

10) What was the result of the first biopsy?
   1. Tissue was normal, the lump or lesion was benign.
   2. There was breast cancer detected
   3. There was carcinoma in situ detected.
   8. DON'T KNOW
   9. REFUSED

11) How old were you when the second biopsy was done?
   888 DON'T KNOW
   999 REFUSED

Employment Questions

Now, just a couple of questions about your work and health insurance.

1) Were you out of work for more than six months during the past two years?
☐ Yes (next question) ☐ No (q.3)
☐ *Don't Know (next question)* ☐ *Refused (q.3)*

2) Why were you out of work? There may be more than one reason listed, so please choose all that apply.
(Show Card 34)
☐ I was retired during this time
☐ I was disabled or on sick leave time
☐ I was caring for a child or infant during this time
☐ I was caring for an elderly person during this time
☐ I was in school during this time
☐ I was in jail or prison during this time
☐ I was not actively seeking employment during this time *(Homemaker)*
☐ I was laid off, dismissed, or looking for employment during this time
☐ *Don't Know (not on card)*
☐ *Refused (not on card)*

3) Which of the following forms of insurance coverage do you have? You may have more than one type of coverage, so please choose all that apply. *(Show Card 35)*
☐ Medicare
☐ Medicaid
☐ Private Insurance–Traditional Indemnity (Aetna, Metropolitan, Prudential etc) or Blue Cross/Shield Plan (e.g. Empire Plan) excluding Community Blue (Blue Cross HMO Plan).
☐ HMO or PPO plan–Health Care Plan, Independent Health, Choice Care, Community Blue, Better Health, Health Source or other.
☐ Veterans Benefits
☐ Worker's Compensation
☐ Other, please list: _____
☐ No Coverage
☐ *Don't Know* ☐ *Refused*

If private insurance selected: Is this your primary health care insurance? ☐ Yes ☐ No
☐ *Don't Know* ☐ *Refused*

4) *(Show Card 36)* How much did you spend out of pocket (that is, not covered by insurance,) last year on health care services (traditional medical care, alternative, non-traditional medical therapy, mental health services, dental services, and/or vision care) for you and your family?
[Other examples: chiropractor, acupuncture, massage therapy (for medical purposes)]
☐ $0 - $250
☐ $251 - $500
☐ $501 - $1,000
☐ $1,001 - $1,500
☐ $1,501 - $2,000

- ☐ $2,001 - $2,500
- ☐ $2,501 - $3,000
- ☐ Over $3,000
- ☐ *Don't Know*
- ☐ *Refused*

5a) How many people are living in your household? (1 = living alone, include partners and dependents) _____

5b) How many individuals not living in your household are financially dependant on the household? _____ (includes those considered as "dependant" by I.R.S. definition such as college students and elderly parents. Does not include those who receive only partial support from the household.)

6) *(Show Card 37)* What is your approximate annual household income (i.e. spouse and other family members contribute to family income)? (select from card)
(categories, 10,000 to 100,000 by 10,000 increments....)

- ☐ <$10,000
- ☐ $10,001 - $20,000
- ☐ $20,001 - $30,000
- ☐ $30,001 - $40,000
- ☐ $40,001 - $50,000
- ☐ $50,001 - $60,000
- ☐ $60,001 - $70,000
- ☐ $70,001 - $80,000
- ☐ $80,001 - $90,000
- ☐ $90,001 - $100,000
- ☐ >$100,000
- ☐ *Don't Know*
- ☐ *Refused*

1. Have you ever had a heart attack or been hospitalized for chest pains or heart problems? (If no, do not ask heart attack)
   ☐ YES ☐ NO ☐ *Don't Know* ☐ *Refused*

2. Please tell me the date of your first heart attack or hospitalization for chest pain or heart problems.
   1. *Less than or equal to 6 months ago (ask triggering questions)*
   2. *More than 6 months ago*
   8. *Don't Know*
   9. *Refused*

3. Have you ever been diagnosed with lung cancer? (If no, ask stem)
   ☐ Yes ☐ No ☐ *Don't Know* ☐ *Refused*

4. I'd like to ask a few questions about treatments you may have received for your lung cancer. Have you received any of the following for your lung cancer? *(Select as many as apply)*
   Surgery, *If yes,* dates:
   Chemotherapy, *If yes,* dates:
   Radiation Therapy, *If yes,* dates:
   Don't Know
   Refused

I am particularly interested in things that may have occurred the two days before your heart attack or hospitalization for chest pains. These next few questions are about the 48 hrs. before your heart attack or heart related event that required hospitalization.

1. *(Show Card 38)* In the 48 hours before your heart attack, did you:
   ☐ Avoid drinking alcoholic beverages
   ☐ Drink less than usual
   ☐ Drink the same as usual
   ☐ Drink more than usual
   ☐ *I don't usually drink*
   ☐ *Don't Know*
   ☐ *Refused*

2. If you avoided drinking or drank less, was it because of the way you were feeling?
   ☐ Yes ☐ No (q.4)
   ☐ *Don't Know (q.4)* ☐ *Refused*

3. *(Show Card 39)* If yes, how were you feeling?
   (check as many as apply)
   ☐ Tired
   ☐ Anxious
   ☐ Nauseous
   ☐ Jittery
   ☐ Lightheaded
   ☐ In pain

- ☐ Dizzy
- ☐ Other: _____
- ☐ No specific feeling
- ☐ Don't Know
- ☐ Was not feeling any different

4. (If MI question 1 answer b or c, Graph of three days, hour by hour time line.)

On what day did you have your heart attack? *(May be day of the week or date of the month.)*

Day of week: XXX
Date: MM-DD-YY

At what time did your heart attack occur?

Time: 00:00

(Leave as 00:00 if Don't Know/Refused)
Completely enter the time, based on a 24-hour clock as shown:

(0000) = Mid
(0100) = 1am
(0200) = 2am
(0300) = 3am
(0400) = 4am
(0500) = 5am
(0600) = 6am
(0700) = 7am
(0800) = 8am
(0900) = 9am
(1000) = 10am
(1100) = 11am
(1200) = Noon
(1300) = 1pm
(1400) = 2pm
(1500) = 3pm
(1600) = 4pm
(1700) = 5pm
(1800) = 6pm
(1900) = 7pm
(2000) = 8pm
(2100) = 9pm
(2200) = 10pm
(2300) = 11pm

Did you have any alcoholic beverages during the two days before or the day of your heart attack? (If yes, continue with charts below.)

(This chart is for showing drinking pattern the day of heart attack)
Please tell me what you had to drink on the day of your heart attack, and at what time you had any drinks.

		B	W	L
(24)	Mid	☐	☐	☐
(1)	1am	☐	☐	☐
(2)	2am	☐	☐	☐
(3)	3am	☐	☐	☐
(4)	4am	☐	☐	☐
(5)	5am	☐	☐	☐
(6)	6am	☐	☐	☐
(7)	7am	☐	☐	☐
(8)	8am	☐	☐	☐
(9)	9am	☐	☐	☐
(10)	10am	☐	☐	☐
(11)	11am	☐	☐	☐

		B	W	L
(12)	Noon	☐	☐	☐
(13)	1pm	☐	☐	☐
(14)	2pm	☐	☐	☐
(15)	3pm	☐	☐	☐
(16)	4pm	☐	☐	☐
(17)	5pm	☐	☐	☐
(18)	6pm	☐	☐	☐
(19)	7pm	☐	☐	☐
(20)	8pm	☐	☐	☐
(21)	9pm	☐	☐	☐
(22)	10pm	☐	☐	☐
(23)	11pm	☐	☐	☐

Enter three digit code for: Beer (#__), Wine (_#_), then Liquor (__#) 0 = None/No More, or enter number of reported drinks of beverage type in appropriate space.

(This chart is for showing drinking pattern 1 day before heart attack.)
Now, please tell me what you had to drink on the day before you had your heart attack, and at what time you had any drinks.

		B	W	L
(24)	Mid	☐	☐	☐
(1)	1am	☐	☐	☐
(2)	2am	☐	☐	☐
(3)	3am	☐	☐	☐
(4)	4am	☐	☐	☐
(5)	5am	☐	☐	☐
(6)	6am	☐	☐	☐
(7)	7am	☐	☐	☐
(8)	8am	☐	☐	☐
(9)	9am	☐	☐	☐
(10)	10am	☐	☐	☐
(11)	11am	☐	☐	☐

		B	W	L
(12)	Noon	☐	☐	☐
(13)	1pm	☐	☐	☐
(14)	2pm	☐	☐	☐
(15)	3pm	☐	☐	☐
(16)	4pm	☐	☐	☐
(17)	5pm	☐	☐	☐
(18)	6pm	☐	☐	☐
(19)	7pm	☐	☐	☐
(20)	8pm	☐	☐	☐
(21)	9pm	☐	☐	☐
(22)	10pm	☐	☐	☐
(23)	11pm	☐	☐	☐

Enter three digit code for: Beer (#__), Wine (_#_), then Liquor (__#) 0 = None/No more, or enter number of reported drinks of beverage type in appropriate space.

(This chart is for showing drinking pattern 2 days before heart attack.)
How about two days before your heart attack, did you have any drinks and at what time?

		B	W	L
(24)	Mid	☐	☐	☐
(1)	1am	☐	☐	☐
(2)	2am	☐	☐	☐
(3)	3am	☐	☐	☐
(4)	4am	☐	☐	☐
(5)	5am	☐	☐	☐
(6)	6am	☐	☐	☐
(7)	7am	☐	☐	☐
(8)	8am	☐	☐	☐
(9)	9am	☐	☐	☐
(10)	10am	☐	☐	☐
(11)	11am	☐	☐	☐

		B	W	L
(12)	Noon	☐	☐	☐
(13)	1pm	☐	☐	☐
(14)	2pm	☐	☐	☐
(15)	3pm	☐	☐	☐
(16)	4pm	☐	☐	☐
(17)	5pm	☐	☐	☐
(18)	6pm	☐	☐	☐
(19)	7pm	☐	☐	☐
(20)	8pm	☐	☐	☐
(21)	9pm	☐	☐	☐
(22)	10pm	☐	☐	☐
(23)	11pm	☐	☐	☐

Enter three digit code for: Beer (#__), Wine (_#_), then Liquor (__#) 0 = None/No More, or enter number of reported drinks of beverage type in appropriate space.

1. Have you ever been diagnosed with breast cancer or diagnosed with carcinoma in situ of the breast (DCIS,LCIS, Ductal carcinoma in situ,or lobular carcinoma in situ)?
   1. YES
   2. NO
   8. DON'T KNOW
   9. REFUSED

I would like to ask you a few questions about treatments you may have received for this breast disease (SHOW CARD 33C).

2. Before your breast disease was diagnosed, what made you think you might have something wrong with your breast? (CHECK AS MANY AS APPLY.)

   ☐ Routine Mammogram
   ☐ Bloody discharge from nipple
   ☐ Non-bloody discharge from nipple
   ☐ Lump in breast discovered by patient
   ☐ Lump in breast discovered by physician
   ☐ Lump under arm discovered by patient
   ☐ Lump under arm discovered by physician
   ☐ Other breast changes noticed by patient
   ☐ Other breast changes noticed by physician
   ☐ Other
   ☐ Don't Know/Unsure
   ☐ REFUSED
   ☐ NONE/NO MORE/NEXT QUESTION

3. What was the month and year that you first thought something was wrong?

   (MM-DD-YY)

4. What surgical treatments did you have? SHOW CARD 33D

   1. Mastectomy (Removal of the entire breast)
   2. Breast Conserving Surgery (Surgery without removal of the entire breast, i.e. lumpectomy, aspiration, partial mastectomy)
   3. No surgery
   4. Other (SPECIFY)
   8. DON'T KNOW/UNSURE
   9. REFUSED

5. Did you have surgery on the lymph nodes under your arm either at the same time as your surgery or in a separate procedure?

   1. YES
   2. NO
   8. DON'T KNOW/NOT SURE
   9. REFUSED

6. Since your breast disease was diagnosed, have you received radiation treatment to the breast or chest?

   1. YES
   2. NO
   8. DON'T KNOW/NOT SURE
   9. REFUSED

7. Is radiation treatment planned to the breast or chest?

   1. YES
   2. NO
   8. DON'T KNOW/NOT SURE
   9. REFUSED

8. Let me ask you about chemotherapy. Not including hormonal therapy, have you received or will you receive chemotherapy, that is given in a hospital or doctor's office by injection or IV?

   1. YES
   2. NO
   8. DON'T KNOW/NOT SURE
   9. REFUSED

9. Are you taking or have you taken tamoxifen (Nolvadex - Hormonal Therapy)?

   1. YES
   2. NO
   3. TOOK FOR A WHILE, NO LONGER TAKING IT.
   4. PLANNING TO TAKE IT, NOT YET BEGUN.
   8. DON'T KNOW/NOT SURE
   9. REFUSED

10. Have you or are you participating in any other Breast Cancer Research studies either for treatment or by being interviewed?

   1. YES
   2. NO

8. DON'T KNOW/NOT SURE
9. REFUSED

10a. What is the name of the study?

10b. When did you start participating in that study?

ENTER START DATE (MM-DD-YY)

10c. When did or will you stop participating in that study?

ENTER ENDING DATE (MM-DD-YY)

If at any time during the administration of these seven questions the respondent VOLUNTEERS that he/she is a casual/social drinker and does not want to continue with this line of questioning, make a note of this under "Notes" and proceed to next section.

1. *(Show Card 40)* The next question is about how frequently you drank alcoholic beverages during the past 12 months. By "drink" we mean either one 12-ounce bottle, can or glass of beer, one wine cooler, one four-ounce glass of wine, one shot of liquor, or one mixed drink containing one shot of liquor. With these definitions in mind, and including any combination of drinks of any kind, what is the largest number of drinks you had in any single day during the past 12 months?
   - A. None (TO NEXT SECTION)
   - B. Between one and three (TO NEXT SECTION)
   - C. Four to ten (TO QUESTION 2)
   - D. Eleven to twenty (TO QUESTION 2)
   - E. Or more than twenty (TO QUESTION 2)
   - *88. Don't Know (TO QUESTION 2)*
   - *99. Refused (TO QUESTION 2?)*

Now I want to ask you just a few questions about the effects of using alcohol in the past year.

2. During the past 12 months, have you often been under the effects of alcohol or suffering its aftereffects while at work or school or while taking care of children?
   - *A. Yes*
   - *B. No*
   - *88. Don't Know*
   - *99. Refused*

3. During the past 12 months, were you ever under the effects of alcohol or feeling its aftereffects in a situation which increased your chances of getting hurt - like when driving a car or boat, using knives or guns or machinery, crossing against traffic, climbing or swimming?
   - *A. Yes*
   - *B. No*
   - *88. Don't Know*
   - *99. Refused*

4. During the past 12 months, did you have any emotional or psychological problems from using alcohol - such as feeling uninterested in things, feeling depressed, suspicious of people, paranoid, or having strange ideas?
   - *A. Yes*
   - *B. No*
   - *88. Don't Know*
   - *99. Refused*

5. During the past 12 months, were you have such a strong desire or urge to use alcohol that you could not resist it or could not think of anything else?
   - *A. Yes*
   - *B. No*
   - *88. Don't Know*
   - *99. Refused*

6. During the past 12 months, did you have a period of a month or more when you spent a great deal of time using alcohol or getting over its effects?
   - *A. Yes*
   - *B. No*
   - *88. Don't Know*
   - *99. Refused*

7. During the past 12 months, did you often use much larger amounts of alcohol than you intended to when you began, or did you use it for a longer period of time than you intended to?
   - *A. Yes*
   - *B. No*
   - *88. Don't Know*
   - *99. Refused*

8. During the past 12 months, did you ever find that you had to use more alcohol than usual to get the same effect or that the same amount had less effect on you than before?
   - *A. Yes*
   - *B. No*
   - *88. Don't Know*
   - *99. Refused*

POST INTERVIEW QUESTIONS

1. *On a scale of 1 to 5, with 1 being the low and 5 being high, in your best judgement, what was this participant's cognitive functioning level?*

   ☐

2. *In your best judgement, was this participant being honest in his/her responses?*

   1) YES
   2) NOT CERTAIN
   3) NO

3. *Was there a specific area or areas that the participant was less than honest?*

   QUESTIONNAIRE SECTIONS
   Physical Activity Sections
   Alcohol Sections
   Tobacco/Smoking Sections
   Reproductive section
   NSAIDS Section
   Oral Section
   Lifetime History Sections
   M.I. Triggering Section
   Alcohol Screening Section
   NONE/NO MORE/NEXT QUESTION

4. *In your best judgement, was this participant RELIABLE in his/her responses?*

   1) YES
   2) NOT CERTAIN
   3) NO

5. *Was there a specific area or areas that the participant was less RELIABLE?*

   QUESTIONNAIRE SECTIONS
   Physical Activity Sections
   Alcohol Sections
   Tobacco/Smoking Sections
   Reproductive section
   NSAIDS Section
   Oral Section
   Lifetime History Sections
   M.I. Triggering Section

Alcohol Screening Section
NONE/NO MORE/NEXT QUESTION

6. *Code hair line loss.*
(SEE INTERVIEWER'S CARD BOOK FOR PICTORIAL EXAMPLES)

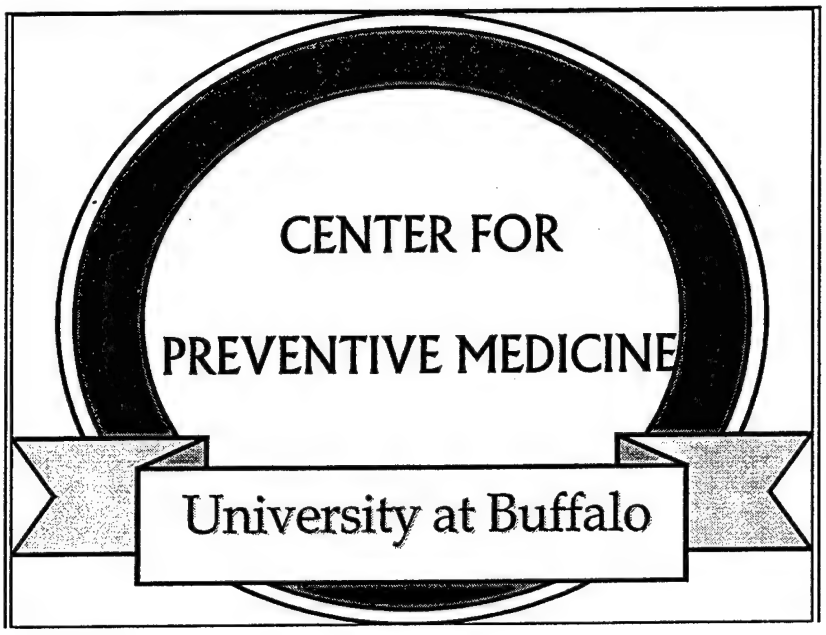
7. *Code right earlobe.*
1) NO CREASE
2) PARTIAL CREASE
3) FULL CREASE
4) DON'T KNOW / UNABLE TO ASSESS

8. *Code left earlobe.*
1) NO CREASE
2) PARTIAL CREASE
3) FULL CREASE
4) DON'T KNOW / UNABLE TO ASSESS

9. *Did participant have evidence of a Dowager's Hump?*
1) YES
2) NO
4) DON'T KNOW / UNABLE TO ASSESS

END OF POST INTERVIEW SECTION

SELF-ADMINISTERED QUESTIONNAIRES



Before your interview appointment:

- Please fill out the enclosed forms, as completely as you can.
- Feel free to check your past records if you need to. Be as accurate as possible, but do NOT worry if you can't recall some things.
- If there are questions you don't understand, leave them blank and we will help you with them at the time of the interview.
- All answers will be kept confidential. Please answer questions honestly.

On the day of your appointment:

- Bring these forms with you to your appointment.
- Please bring the bottles or containers for all medicines you've taken in the last 30 days. Bring medicines prescribed by a doctor and medicines you get without a prescription, including pain pills, vitamins, supplements, antacids and sleeping aids.
- Please wear comfortable, loose-fitting clothing. If possible, do not wear pantyhose, girdles, bodysuits or similar undergarments. If you wear a heavy sweatshirt or sweater, please wear a thin shirt or t-shirt underneath it.
- **If you are scheduled to have blood drawn, please do not eat or drink between the time of your evening meal and the time of your appointment. We will provide a light breakfast.**

Thank you for your help. We appreciate it, and are looking forward to seeing you soon.

Revised: July 7, 1999

PART I SELF ADMINISTERED QUESTIONNAIRE

Please fill in the blanks or check the box that indicates your answer to each question.

1a. Please print your name, current address and telephone number.

Name: _____

Address: _____

_____

Telephone number: Area code ( ) _____

1b. Do you have a different address part of the year (for example a winter address, or living with a relative for a few months each year?) ☐ **Yes**↓ ☐ No →Please go to question 1c.

IF YES, please print your part-year address and telephone number:

Address: _____
Number Street

_____
City State Zip

Telephone Number: Area Code ( ) _____

1c. What was your name at birth? _____ _____ _____
Last First Middle

1d. What was your mother's maiden name?

_____ _____ _____
Last First Middle

2a. Are you planning to move in the next 3 years?

☐ No ☐ Yes: Where _____

2b. Please give us the name of a relative or friend, not living in your household, who would be likely to know how to contact you if we cannot contact you directly:
(This person does not need to live in Western New York.)

Name: _____

Address: _____

_____

Telephone number: Area Code ( ) _____

Relationship: _____

3. What is your date of birth? ☐☐ Month ☐☐ Day ☐☐☐☐ Year

4. What is your sex:
   2-☐ Male
   1-☐ Female

5. How would you describe your racial or ethnic group? If you are of mixed blood, which group do you identify with most?
   1-☐ American Indian or Alaskan Native
   2-☐ Asian or Pacific Islander (ancestry is Chinese, Indo-Chinese, Korean, Japanese, Pacific Islander, or Vietnamese)
   3-☐ Black or African-American (not of Hispanic origin)
   4-☐ Hispanic/Latino (ancestry is Mexican, Cuban, Puerto Rican, Central American, or South American)
   5-☐ White (not of Hispanic/Latino origin)
   6-☐ Other (specify_____)

6. What was the highest grade or year of school that you completed? Include trade or vocational school that was not part of high school.
   1-☐ Less than 12 years of school (No high school diploma)
   ↓
   Highest grade completed: ☐☐
   2-☐ High school diploma/certificate
   3-☐ GED-General Equivalency Diploma
   4-☐ Vocational/Technical certificate WITHOUT HS Diploma/GED
   5-☐ Vocational/Technical certificate AND HS Diploma/GED
   6-☐ Some college
   7-☐ Associate Degree
   8-☐ Bachelor's Degree
   9-☐ Graduate Degree (i.e. MS, PHD, MD, LLB)

7. What is your present marital status?
   1-☐ Never Married
   2-☐ Married
   3-☐ Widowed
   4-☐ Divorced/Separated
   5-☐ Living with Someone as if Married

12. These questions are about your medical history. **If you have been told by a doctor or health care professional that you have or have had any of the listed conditions, please check "Yes" and fill in the other items.** Check "No" if you have never been told that you have the condition.

Condition	(0) No	(1) Yes →	If yes, Date First Diagnosed
High blood pressure (hypertension)	☐No	☐Yes	__/__/__
Was the high blood pressure treated by medication(s)?	☐No ☐Don't Know	☐Yes	
High blood cholesterol	☐No	☐Yes	__/__/__
Was the high blood cholesterol treated by medication(s)?	☐No ☐Don't Know	☐Yes	
Diabetes (not during pregnancy)	☐No	☐Yes	__/__/__
Was this ☐ Non-Insulin Dependent Diabetes Or ☐ Insulin Dependent Diabetes			
High or elevated sugar in blood or urine	☐No	☐Yes	__/__/__
Angina (chest pain related to your heart)	☐No	☐Yes	__/__/__
If yes, was the angina confirmed by angiogram?	1-☐No 3-☐Don't Know	2-☐Yes	
Heart attack (myocardial infarction, MI)	☐No	☐Yes	__/__/__
Number of times this occurred _____			
Atrial fibrillation (special type of irregular heart beat)	☐No	☐Yes	__/__/__
Irregular heart beat (arrhythmia)	☐No	☐Yes	__/__/__
Diseased heart valve	☐No	☐Yes	__/__/__
Rheumatic heart disease	☐No	☐Yes	__/__/__
Stroke	☐No	☐Yes	__/__/__
Number of times this occurred _____			
Peripheral vascular disease (intermittent claudication or leg pain on exercise, but not varicose veins)	☐No	☐Yes	__/__/__
Deep venous thrombosis (blood clots in your legs, but not varicose veins)	☐No	☐Yes	__/__/__
Aortic aneurysm (blood clot in the big artery going to the heart)	☐No	☐Yes	__/__/__
Pulmonary embolus (blood clot in the lung)	☐No	☐Yes	__/__/__

Other Conditions	(0) No	(1) Yes
Chronic bronchitis	☐No	☐Yes
Gall bladder disease	☐No	☐Yes
Kidney or bladder stones	☐No	☐Yes
Kidney disease	☐No	☐Yes
Hepatitis (jaundiced)	☐No	☐Yes
Liver cirrhosis	☐No	☐Yes
Polyps in your colon or rectum	☐No	☐Yes
Broken bones as an adult (includes stress fractures) **If yes, please specify which bone, date of fracture and how fracture occurred:** **bone:**_____ **Year:**_____ **How happened**_____ **bone:**_____ **Year:**_____ **How happened**_____ **bone:**_____ **Year:**_____ **How happened**_____ **bone:**_____ **Year:**_____ **How happened**_____	☐No	☐Yes
Osteoporosis (thinning bones)	☐No	☐Yes
Seizures	☐No	☐Yes
Depression	☐No	☐Yes
Any other disease: _____	☐No	☐Yes

Your Doctor's Name: _____

Address: _____

_____

_____

Procedure	(0) No	(1) Yes ☞	Date of Most Recent
Chest x-ray If yes, how many _____	☐No	☐Yes	___/___/___
X-ray of the spine or back About how many **back x-rays** have you had in your life: _____	☐No	☐Yes	___/___/___
Dental x-ray If yes, about how many dental x-rays have you had in your life: _____	☐No	☐Yes	___/___/___
Other x-ray/Radiation treatment (not diagnostic): _____	☐No	☐Yes	___/___/___
Bronchoscopy (exam of your lungs with a small scope)	☐No	☐Yes	___/___/___

WOMEN ONLY:

Procedure	(0) No	(1) Yes ☞	Date of Last Procedure	How many times in the past 5 years?
Pap test	☐No	☐Yes	___/___/___	
Breast exam by clinician	☐No	☐Yes	___/___/___	
Mammogram	☐No	☐Yes	___/___/___	
Breast Biopsy	☐No	☐Yes	___/___/___	

If yes, how was the breast lump initially found?
☐ Breast self-exam
☐ Physician's exam
☐ Mammography
☐ Other, please describe: _____

Where was breast biopsy performed?
Doctor/Clinic: _____

Street: _____

City, State: _____

LIFETIME PHYSICAL ACTIVITY

In this section, we are interested in your lifetime physical activities, starting at age 10. These activities may include sports play, other leisure time activity or exercise, and household chores or manual labor activities.

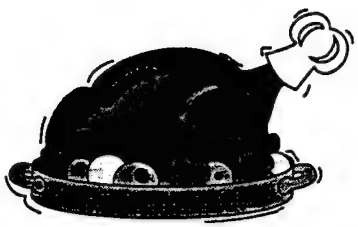
16. How often did you regularly do any **sports** (such as basketball, gymnastics, or field hockey) and/or **other strenuous leisure time physical activity or exercise** (such as dancing (ballet, tap, etc.), hiking, jogging, biking, swimming, etc.), **vigorous household chores** (such as scrubbing floors, vacuuming, moving furniture, washing windows, etc.) or **manual yard work** (such as digging with a shovel, mowing lawn with a hand mower, carrying, chopping or stacking wood, shoveling snow, etc.) during each age period listed below? (Please check the appropriate box for each age period.)

Age Period	0 Hours/Week	< 1 hour Per Week	1-3 Hours/Week	> 3 to 6 Hours/Week	Over 6 Hours/Week
10-13 Years of age (before high school)	☐	☐	☐	☐	☐
14-18 Years of age (during high school)	☐	☐	☐	☐	☐
19-22 Years of age	☐	☐	☐	☐	☐
23-34 Years of age	☐	☐	☐	☐	☐
35-50 Years of age	☐	☐	☐	☐	☐
51-64 Years of age	☐	☐	☐	☐	☐
65+ Years of age	☐	☐	☐	☐	☐
CODES	**(1)**	**(2)**	**(3)**	**(4)**	**(5)**

17. For each of the following age periods, mark whether your physical activity level was the same, more active or less active than others your age and sex:

	Much More Active	More Active	The Same	Less Active	Much Less Active
10-13 Years of age (before high school)	☐	☐	☐	☐	☐
14-18 Years of age (during high school)	☐	☐	☐	☐	☐
19-22 Years of age	☐	☐	☐	☐	☐
23-34 Years of age	☐	☐	☐	☐	☐
35-50 Years of age	☐	☐	☐	☐	☐
51-64 Years of age	☐	☐	☐	☐	☐
65+ Years of age	☐	☐	☐	☐	☐
CODES	**(1)**	**(2)**	**(3)**	**(4)**	**(5)**

	Medium Serving	Your Serving Size			How Often?								
		S	M	L	Rarely or Never	1 per mo	2-3 per mo	1 per wk	2 per wk	3-4 per wk	5-6 per wk	1 per day	2+ per day
FRUITS & JUICES													
EXAMPLE: Apples, Applesauce, pears	(1) or 1/2 cup		✓					✓					
Apples, applesauce, pears	(1) or 1/2 cup												
Bananas	1 medium												
Peaches, apricots (canned, frozen or dried, whole year)	(1) or 1/2 cup												
Peaches, apricots, nectarines (fresh, in season)	1 medium												
Cantaloupe (in season)	1/4 medium												
Watermelon (in season)	1 slice												
Strawberries (fresh, in season)	1/2 cup												
Oranges	1 medium												
Orange juice or grapefruit juice	6 oz. glass												
Grapefruit	(1/2)												
Tang, Hi-C	6 oz. glass												
Other fruit juices, fortified fruit drinks	6 oz. glass												
Any other fruit, including berries, fruit cocktail	1/2 cup												
VEGETABLES													
String beans, green beans	1/2 cup												
Peas	1/2 cup												
Chili with beans	3/4 cup												
Other beans such as baked beans,pintos,kidney beans,limas	3/4 cup												
Corn	1/2 cup												
Winter squash such as acorn, butternut	1/2 cup												
Summer squash, zucchini, okra	1/2 cup												
CODES		(1)	(2)	(3)	1	2	3	4	5	6	7	8	9



	Medium Serving	Your Serving Size			How often?								
		S	M	L	Rarely or Never	1 per mo	2-3 per mo	1 per wk	2 per wk	3-4 per wk	5-6 per wk	1 per day	2+ per day
MEAT, FISH, POULTRY & MIXED DISHES													
Hamburgers, cheeseburgers, meat loaf	1 medium												
Beef (steaks, roasts) or lamb	4 oz.												
Beef stew or pot pie with carrots, other vegetables	1 cup												
Liver, including chicken livers	4 oz.												
Pork, including chops, roasts, ham	2 chops or 4 oz.												
Fried chicken	2 small or 1 large piece												
Chicken or turkey, roasted, stewed or broiled	2 small or 1 large piece												
Chicken wings	6 pieces												
Fried fish or fish sandwich	4 oz or 1 sandwich												
Tuna fish, tuna salad, tuna casserole	1/2 cup												
Shell fish (shrimp, lobster, crab, oysters, etc.)	(5) 1/4 cup or 3 oz.												
Other fish, broiled, baked	4 oz.												
Spaghetti, lasagna, other pasta with tomato sauce	1 cup												
Pizza	2 slices												
Mixed dishes with cheese (such as macaroni and cheese)	1 cup												
LUNCH ITEMS													
Liverwurst	2 slices												
Hot Dogs	2 dogs												
Ham, lunch meats such as bologna, salami	2 slices												
Vegetable soup, vegetable beef, minestrone, tomato soup	1 medium bowl												
Other soups	1 medium bowl												
CODES		(1)	(2)	(3)	1	2	3	4	5	6	7	8	9

	Medium Serving	Your Serving Size			How often?								
		S	M	L	Rarely or Never	1 per mo	2-3 per mo	1 per wk	2 per wk	3-4 per wk	5-6 per wk	1 per day	2+ per day
SWEETS													
Ice cream	1 scoop												
Doughnuts, cookies, cakes, pastry	1 piece or 3 cookies												
Pumpkin pie, sweet potato pie	1 medium slice												
Other pies	1 medium slice												
Chocolate candy and candy bars	small bar, 1 oz.												
Other candy, jelly, honey, brown sugar, syrup	3 pieces or 1 Tablespn.												
DAIRY PRODUCTS													
Cottage cheese	1/2 cup												
Other cheeses and cheese spreads	2 slices or 2 oz.												
Flavored yogurt	1 cup												
Whole milk and beverages with whole milk (not incl. on cereal)	8 oz. glass												
2% milk and beverages with 2% milk (not incl. on cereal)	8 oz. glass												
Skim milk, 1% milk or buttermilk (not incl. on cereal)	8 oz. glass												
BEVERAGES													
Regular soft drinks	12 oz. can or bottle												
Diet soft drinks	12 oz. can or bottle												
Beer	12 oz. can or bottle												
Wine	1 medium glass												
Liquor	1 shot												
Decaffeinated coffee	1 medium cup												
Coffee, not decaffeinated	1 medium cup												
CODES		(1)	(2)	(3)	1	2	3	4	5	6	7	8	9

19. Think about your diet over the period **12 to 24 months ago** and the responses you have just made on this questionnaire. Are there any foods not mentioned which you ate *at least once a week*, even in small quantities, or ate frequently in a particular season? Consider other meats, breakfast foods, catsup, green chilies or jalapeños, avocado (guacamole), Mexican dishes, Chinese or other ethnic foods, other fruits or vegetables, as well as nutritional supplements (bran, etc.).

FOOD:	Your Serving Size			How often?								
	S	M	L	Rarely or Never	1 per mo	2-3 per mo	1 per wk	2 per wk	3-4 per wk	5-6 per wk	1 per day	2+ per day
CODES	(1)	(2)	(3)	1	2	3	4	5	6	7	8	9

20. Were you on a special diet 12-24 months ago? (Check all that are true.)

☐ No ----------> (go to next page.)

☐ Weight Loss
☐ Weight Gain
☐ Low Salt
☐ Vegetarian
☐ For Medical Condition
☐ Diabetic Diet
☐ Low Fat
☐ Low Cholesterol
☐ Other: _____
_____

↓

20a. Did a doctor or nurse tell you to go on this special diet?
1-☐ Yes
0-☐ No

20b. When did you begin this special diet?

_____/19_____
month year

26. How often did you have pan fried, sauteed or wok cooked foods, such as onions, fish or chicken?

☐ A. Never	**CODE = 1**
☐ B. One or less times a month	**CODE = 2**
☐ C. Once a week	**CODE = 3**
☐ D. 2 times per week	**CODE = 4**
☐ E. 3 to 4 times per week	**CODE = 5**
☐ F. 5 to 6 times per week	**CODE = 6**
☐ G. Once a day	**CODE = 7**
☐ H. 2 or more times per day	**CODE = 8**
☐ I. Don't know	**CODE = 88**

27. During the time 12 to 24 months ago (between 1 to 2 years ago), what kinds of fat or oil were usually used to deep fry, pan fry, or saute the foods you ate? (select up to three types)

☐ Butter
☐ Low calorie margarine
☐ Stick margarine
☐ Tub margarine
☐ Solid vegetable fat
☐ Shortening (lard, bacon fat, drippings, salt pork or ham hock)
☐ Olive Oil
☐ Canola Oil
☐ Peanut Oil
☐ Other vegetable oils (corn, saffron, sunflower)
☐ Non-stick spray (e.g. Pam)
☐ Didn't use fat
☐ Don't know

28. How often did you have beans, rice, vegetables and potatoes cooked with fat or oil?

☐ A. Never	**CODE = 1**
☐ B. One or less times a month	**CODE = 2**
☐ C. Once a week	**CODE = 3**
☐ D. 2 times per week	**CODE = 4**
☐ E. 3 to 4 times per week	**CODE = 5**
☐ F. 5 to 6 times per week	**CODE = 6**
☐ G. Once a day	**CODE = 7**
☐ H. 2 or more times per day	**CODE = 8**
☐ I. Don't know	**CODE = 88**

29. During the time 12 to 24 months ago, what kinds of fat or oil did you usually use <u>when cooking</u> beans, rice, vegetables and potatoes? (select up to three types)

☐ Butter
☐ Low calorie margarine
☐ Stick margarine
☐ Tub margarine
☐ Solid vegetable fat
☐ Shortening (lard, bacon fat, drippings, salt pork or ham hock)
☐ Olive Oil
☐ Canola Oil
☐ Peanut Oil
☐ Other vegetable oils (corn, saffron, sunflower)
☐ Non-stick spray (e.g. Pam)
☐ Didn't use fat
☐ Don't know

34. How often did you eat the skin of your chicken or turkey?

☐ A. Always	**CODE = 1**
☐ B. Very Often	**CODE = 2**
☐ C. Sometimes	**CODE = 3**
☐ D. Never	**CODE = 4**
☐ E. Don't eat chicken or turkey	**CODE = 5**
☐ F. Don't know	**CODE = 8**

35. How often did you eat the fat on meat such as beef, veal or pork, including roasts and hams?

☐ A. Always	**CODE = 1**
☐ B. Very Often	**CODE = 2**
☐ C. Sometimes	**CODE = 3**
☐ D. Never	**CODE = 4**
☐ E. Don't eat beef, veal or pork	**CODE = 5**
☐ F. Don't know	**CODE = 8**

36. How often did you eat foods which were grilled or broiled?

☐ A. Never or less than once per month	**CODE = 1**
☐ B. 1 - 3 times per month	**CODE = 2**
☐ C. Once per week	**CODE = 3**
☐ D. 2 -4 times per week	**CODE = 4**
☐ E. 5-6 times per week	**CODE = 5**
☐ F. 7 or more times per week	**CODE = 6**

37. When you ate meat, how well done was the meat?

1-☐ rare
2-☐ medium rare
3-☐ medium
4-☐ medium well
5-☐ well

38. Did you avoid drinking milk because drinking milk caused any of the following?
(Select as many as apply.)

☐ Stomach growling
☐ Stomach distention
☐ Gas
☐ Diarrhea
☐ No particular reason
☐ I have no problems drinking milk
☐ Other: _____

This completes the initial take-home part of the study. Thank you for completing this packet.

At the time of your interview appointment you will be asked questions about a number of topics including alcohol use, smoking history, and exposure to passive smoke (second hand smoke) during different periods of your life. Please use the enclosed Lifetime Events Calendar form to help you remember what was happening in your life during different time periods.

One section of the interview will ask you about the number of hours you currently spend in various types of activities during the work week and on weekends. Please take a few minutes before your interview appointment to think about how you spend your time during the week and on weekends. Please consider household, occupational, and leisure activities.

Go back to birth year and place.

12. **Residential History** Please list each place you have lived up to now, starting with your current home and working backwards; include any places you lived during school and/or military service. Include every place you have lived, including every address, even if it was in the same town. Please see example below.

Number, Street & Apt #	City, Town, Village or Nearest Village	State and Zip Code	Year of move to this location	Year of leaving this location	Number of individuals living in household (1 = Live by yourself)	Number of individuals living in the household who smoked
EXAMPLE: 123 Main Street, Apt. 8	Buffalo	NY 14203	1987	1995	3	1
1.						
2.						
3.						
4.						
5.						
6.						
7.						
8.						
9.						
10.						

CONTINUED ON OTHER SIDE
→ → → → → → → → → → → →

We are interested in the brands of cigarettes that you have smoked during your lifetime. Please check your usual brand of cigarettes for each of the age periods.

When you were **20 years old and younger**, what brand did you usually buy?

☐ Did not smoke during this age period

☐ Benson & Hedges
☐ Camel
☐ Carlton
☐ Chesterfield
☐ Generic
☐ Kent
☐ Kool
☐ L & M
☐ Lark
☐ Lucky Strikes
☐ Marlboro
☐ Merit
☐ More
☐ Newport
☐ Old Gold
☐ Other
☐ Pall Mall
☐ Parliament
☐ Phillip Morris
☐ Raleigh
☐ Salem
☐ Tareyton
☐ True
☐ Vantage
☐ Viceroy
☐ Virginia Slim
☐ Winston

Between the **ages of 21 and 30**, what brand did you usually buy?

☐ Did not smoke during this age period

☐ Benson & Hedges
☐ Camel
☐ Carlton
☐ Chesterfield
☐ Generic
☐ Kent
☐ Kool
☐ L & M
☐ Lark
☐ Lucky Strikes
☐ Marlboro
☐ Merit
☐ More
☐ Newport
☐ Old Gold
☐ Other
☐ Pall Mall
☐ Parliament
☐ Phillip Morris
☐ Raleigh
☐ Salem
☐ Tareyton
☐ True
☐ Vantage
☐ Viceroy
☐ Virginia Slim
☐ Winston

CONTINUED ON OTHER SIDE
→ → → → → → → → → → → →

Between the **ages of 51 and 60**, what brand did you usually buy?

☐ Did not smoke during this age period

☐ Benson & Hedges
☐ Camel
☐ Carlton
☐ Chesterfield
☐ Generic
☐ Kent
☐ Kool
☐ L & M
☐ Lark
☐ Lucky Strikes
☐ Marlboro
☐ Merit
☐ More
☐ Newport
☐ Old Gold
☐ Other
☐ Pall Mall
☐ Parliament
☐ Phillip Morris
☐ Raleigh
☐ Salem
☐ Tareyton
☐ True
☐ Vantage
☐ Viceroy
☐ Virginia Slim
☐ Winston

Between the **ages of 61 to your current age**, what brand did you usually buy?

☐ Did not smoke during this age period

☐ Benson & Hedges
☐ Camel
☐ Carlton
☐ Chesterfield
☐ Generic
☐ Kent
☐ Kool
☐ L & M
☐ Lark
☐ Lucky Strikes
☐ Marlboro
☐ Merit
☐ More
☐ Newport
☐ Old Gold
☐ Other
☐ Pall Mall
☐ Parliament
☐ Phillip Morris
☐ Raleigh
☐ Salem
☐ Tareyton
☐ True
☐ Vantage
☐ Viceroy
☐ Virginia Slim
☐ Winston

HEALTH STATUS QUESTIONNAIRE

This survey asks for your views about your health. Answer every question by checking the appropriate box...If you are unsure about how to answer a question, please give the best answer you can and make a comment in the left margin.

1. In general, would you say your health is:
   - ☐ Excellent
   - ☐ Very good
   - ☐ Good
   - ☐ Fair
   - ☐ Poor

2. Compared to one year ago, how would you rate your health in general now?
   - ☐ Much better now than one year ago
   - ☐ Somewhat better now than one year ago
   - ☐ About the same
   - ☐ Somewhat worse now than one year ago
   - ☐ Much worse now than one year ago

The following items are about activities you might do during a typical day. Does your health now limit you in these activities? If so, how much?

		Yes, limited a lot	Yes, limited a little	No, not limited at all
3.	Vigorous activities, such as running, lifting heavy objects, participating in strenuous sports	☐	☐	☐
4.	Moderate activities, such as moving a table, pushing a vacuum cleaner, bowling, or playing golf	☐	☐	☐
5.	Lifting or carrying groceries	☐	☐	☐
6.	Climbing several flights of stairs	☐	☐	☐
7.	Climbing one flight of stairs	☐	☐	☐
8.	Bending, kneeling, or stooping	☐	☐	☐
9.	Walking more than a mile	☐	☐	☐
10.	Walking several blocks	☐	☐	☐
11.	Walking one block	☐	☐	☐
12.	Bathing or dressing yourself	☐	☐	☐

CONTINUED ON OTHER SIDE
→ → → → → → → → → → → →

These questions are about how you feel and how things have been with you during the past 4 weeks. For each question, please give the one answer that comes closest to the way you have been feeling.

How much of the time during the past 4 weeks...

		All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	None of the time
23.	Did you feel full of pep?	☐	☐	☐	☐	☐	☐
24.	Have you been a very nervous person?	☐	☐	☐	☐	☐	☐
25.	Have you felt so down in the dumps that nothing could cheer you up?	☐	☐	☐	☐	☐	☐
26.	Have you felt calm and peaceful?	☐	☐	☐	☐	☐	☐
27.	Did you have a lot of energy?	☐	☐	☐	☐	☐	☐
28.	Have you felt downhearted and blue?	☐	☐	☐	☐	☐	☐
29.	Did you feel worn out?	☐	☐	☐	☐	☐	☐
30.	Have you been a happy person?	☐	☐	☐	☐	☐	☐
31.	Did you feel tired?	☐	☐	☐	☐	☐	☐

CONTINUED ON OTHER SIDE
→ → → → → → → → → → → → →

WE NEED TO KNOW HOW YOU HAVE BEEN FEELING

Check the box for each statement which best describes how often you felt or behaved this way DURING THE PAST WEEK.

DURING THE PAST WEEK:		Rarely or None of the time (Less than 1 Day)	Some of the Time (1-2 Days)	Occasionally (3-4 Days)	Most or All of the time (5-7 Days)
a.	I was bothered by things that usually don't bother me	☐	☐	☐	☐
b.	I did not feel like eating: my appetite was poor	☐	☐	☐	☐
c.	I felt that I could not shake off the blues even with help of my family and friends	☐	☐	☐	☐
d.	I felt that I was just as good as other people	☐	☐	☐	☐
e.	I had trouble keeping my mind on what I was doing	☐	☐	☐	☐
f.	I felt depressed	☐	☐	☐	☐
g.	I felt that everything I did was an effort	☐	☐	☐	☐
h.	I felt hopeful about the future	☐	☐	☐	☐
i.	I thought my life had been a failure	☐	☐	☐	☐
j.	I felt fearful	☐	☐	☐	☐
k.	My sleep was restless	☐	☐	☐	☐
l.	I was happy	☐	☐	☐	☐
m.	I talked less than usual	☐	☐	☐	☐
n.	I felt lonely	☐	☐	☐	☐
o.	People were unfriendly	☐	☐	☐	☐
p.	I enjoyed life	☐	☐	☐	☐
q.	I had crying spells	☐	☐	☐	☐
r.	I felt sad	☐	☐	☐	☐
s.	I felt that people disliked me	☐	☐	☐	☐
t.	I could not get "going"	☐	☐	☐	☐

In this section, we want to know about **your family and your family's health.**

These questions are only for your first degree relatives. These include your mother, father, sisters, brothers, half-brothers, half-sisters, sons and daughters. Please do not include adopted parents, adopted brothers or sisters, adopted children or step-brothers or step-sisters.

I am adopted and do not know about my parents and or my sibling's health. ____ True

	Year of Birth?	Still Living?		Year of Death?	Age at Death	Cause of Death?
		Yes	No ☞			
Mother						
Father						
Brother #1						
Brother #2						
Brother #3						
Brother #4						
Brother #5						
Brother #6						
Brother #7						
Brother #8						
Brother #9						
Sister #1						
Sister #2						
Sister #3						
Sister #4						
Sister #5						
Sister #6						
Sister #7						
Sister #8						
Sister #9						

CONTINUED ON OTHER SIDE
→ → → → → → → → → → → → →

These next few questions are about health problems among your relatives.

1. Was/Were (any of) your _____ ever diagnosed with **breast cancer**?

Relationship	Age Diagnosed
☐ Mother	_____
☐ Sister	_____
☐ Sister	_____
☐ Half-sister	_____
☐ Half-sister	_____
☐ Daughter	_____
☐ Daughter	_____

☐ No breast cancer among these relatives.

2. Were any of your relatives ever diagnosed with **any other type of cancer**?

Relationship	Primary/first Cancer Site	Age Diagnosed
☐ Mother	_____	_____
☐ Father	_____	_____
☐ Sister	_____	_____
☐ Sister	_____	_____
☐ Half-sister	_____	_____
☐ Half-sister	_____	_____
☐ Brother	_____	_____
☐ Brother	_____	_____
☐ Half-brother	_____	_____
☐ Half-brother	_____	_____
☐ Daughter	_____	_____
☐ Daughter	_____	_____
☐ Son	_____	_____
☐ Son	_____	_____

☐ No cancer among these relatives.

CONTINUED ON OTHER SIDE
→ → → → → → → → → → → →

4. Were any of your relatives ever diagnosed with **diabetes or high blood sugar**?

Relationship	Age Diagnosed
☐ Mother	_____
☐ Father	_____
☐ Sister	_____
☐ Sister	_____
☐ Sister	_____
☐ Half-sister	_____
☐ Half-sister	_____
☐ Brother	_____
☐ Brother	_____
☐ Brother	_____
☐ Half-brother	_____
☐ Half-brother	_____
☐ Daughter	_____
☐ Daughter	_____
☐ Daughter	_____
☐ Son	_____
☐ Son	_____
☐ Son	_____

☐ No diabetes or high blood sugar among these relatives.

CONTINUED ON OTHER SIDE
→ → → → → → → → → → → →

6. Please think now about some of your other blood relatives (aunts, uncles, grandparents, grandchildren, nieces and nephews). Were any of these relatives ever diagnosed with cancer?

☐ Yes ☐ No

If yes, please tell us which relatives had the disease and what kind of cancer it was:

_____

_____

_____

_____

In that same group of blood relatives, were any of them diagnosed with heart disease?

☐ Yes ☐ No

If yes, please tell us which relatives had the disease and what kind of heart disease it was:

_____

_____

_____

_____

7. Do **any other diseases or conditions** tend to run in your family?

☐ Yes ☐ No

If yes, please explain and tell us which relatives had the disease or condition:

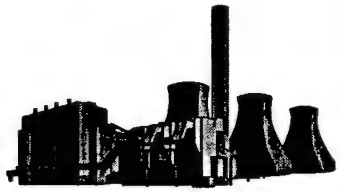
_____

_____

_____

_____

In your work, are (were) you ever exposed to any of the following? If you were usually or occasionally exposed, indicate the calendar years you were exposed and the kind of protective devices present.

Exposure 	(check one)				Calendar Year Exposed	Did You Wear a Mask or Respirator? (Check one)			Did the Work-site Have an Exhaust Vent System? (check one)		
	Never	Some of the time ->	Most of the time ->	Don't Know	19__ to 19__	No	Yes	Some of the time	No	Yes	Don't Know
Organic Compounds:											
Acrylonitrile											
Carbon Black											
Chloromethyl Ethers											
Coal Tar/Pitch/Asphalt											
Diesel Exhaust											
Dyes											
Gasoline/Oil											
Pesticides/Insecticides											
Plastics											
Polychlorinated Biphenyls (PCBs)											
Solvents											
Vinyl Chloride											

CONTINUED ON OTHER SIDE
→ → → → → → → → → → → →

Please check whether you ever worked in any of the following jobs or industries. If you worked in any of these jobs, please write the month and year you began the job and ended the job.

Type of Job or Industry	Check one:		If Yes:	
	No	Yes →	Month/Year Started	Month/Year Ended
Battery Production Worker				
Coke-Oven Worker				
Foundry Worker				
Iron or Steel Plant Worker				
Rubber or Tire Making Worker				
Chemical Plant Worker				
Oil Refinery Worker				
Auto Mechanic				
Insulation Worker				
Leather Tanning Worker				
Meat Packer/Butcher				
Miner				
Painter				
Pipe-fitter, Plumber or Boilermaker				
Roofer				
Ship Builder				
Shoemaker or Shoe Repair Worker				
Truck Driver				
Electroplate Worker				
Smelt Worker				
Metal Refining				
Welder				
Solderer				
Chemist				
Laboratory Worker				

CONTINUED ON OTHER SIDE
→ → → → → → → → → → → →

Occupational History In this section we ask you to answer questions about all the jobs you've worked for at least a year, whether full or part-time. Please start with your current or most recent job. For each employer provide all of your job titles. Be as specific and complete as possible. For example if your job was "engineer" tell us what kind - civil, stationary, or electrical, "clerk" tell us what type - sales, or shipping, "teacher" tell us - elementary, or high school. Fill out each section as completely as you can.

Example #1:

The name and location of company you worked for? HAPPY MOTORS, INC.
BUFFALO, NY

What was your job title there? If more than one, list all titles.

1) DRILL MACHINE OPERATOR
2) HONING MACHINE OPERATOR
3) LOADER, HEAT TREAT. FURNACE
4) DRIVER, POWER TRUCK, INSIDE

What did this company (employer) manufacture or do?
MACHINED & ASSEMBLED CAR & TRUCK ENGINES.

Were there co-workers who smoked cigarettes near you, so that you frequently breathed their smoke?

☑ No (next page) ☐ Yes →

For how many hours a day were you exposed to this smoke? ☐☐

For how many years were you exposed to this smoke? ☐☐

Describe your activities and duties for each job title, listing the calendar years you did this work.

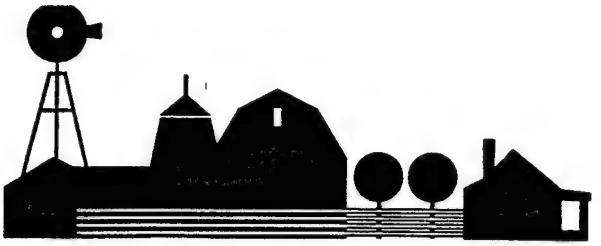
1) 19 6 0 to 19 7 0 DRILLED PARTS USING MACHINE OILS

2) 19 7 0 to 19 7 7 MACHINED ENGINE PARTS WITH MINERAL OILS

3) 19 7 7 to 19 8 0 LOADED OVEN/FURNACE WITH MANIFOLDS

4) 19 8 0 to 19 8 5 DROVE FORKLIFT TRUCK RETIRED 1985

CONTINUED ON OTHER SIDE
→ → → → → → → → → → → →



Occupational History (continued)

Current or most recent:

The name and location of company you worked for?	
What was your job title there? If more than one, list all titles. 1) 2) 3) 4)	
What did this company (employer) manufacture or do?	
Were there co-workers who smoked cigarettes near you, so that you frequently breathed their smoke? ☐ No (next page) ☐ Yes →	For how many hours a day were you exposed to this smoke? ☐☐ For how many years were you exposed to this smoke? ☐☐

Describe your activities and duties for each job title, listing the calendar years you did this work.

1) 19☐☐ to 19☐☐ _____

_____

2) 19☐☐ to 19☐☐ _____

_____

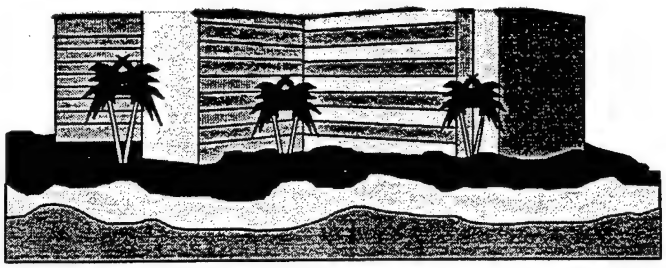
3) 19☐☐ to 19☐☐ _____

_____

4) 19☐☐ to 19☐☐ _____

_____

CONTINUED ON OTHER SIDE
→ → → → → → → → → → → →



Occupational History (continued)

Next most recent:

The name and location of company you worked for?

What was your job title there? If more than one, list all titles.

1)

2)

3)

4)

What did this company (employer) manufacture or do?

Were there co-workers who smoked cigarettes near you, so that you frequently breathed their smoke?

☐ No (next page) ☐ Yes →

For how many hours a day were you exposed to this smoke? ☐☐

For how many years were you exposed to this smoke? ☐☐

Describe your activities and duties for each job title, listing the calendar years you did this work.

1) 19☐☐ to 19☐☐ _____

_____

2) 19☐☐ to 19☐☐ _____

_____

3) 19☐☐ to 19☐☐ _____

_____

4) 19☐☐ to 19☐☐ _____

_____

PROTOCOL FOR SPECIMEN STORAGE

Cryogenic storage system and collected data description

Table 1: Number of participants with samples stored in Biological Specimen Bank

Category	Participants	Percentage
Case	1803	32.08%
Control	3817	67.92%
	5620	

Most samples are stored in liquid nitrogen (-196 C). These samples include: serum, heparin plasma, Citrate plasma, buffy coat, and red blood cells.

Table 2: Samples stored in liquid nitrogen

Category	S	HP	CP	BC	RBC
Case	12645	9875	10021	6748	6695
Control	28094	22076	22494	15072	14930
	40739	**31951**	**32515**	**21820**	**21625**

Key
S: Serum
HP: Heparin Plasma
CP: Citrate Plasma
BC: Buffy Coat
RBC: Red Blood Cells

In addition there are samples stored in -80 degree freezers. Table 3 describes these samples.

Table 3: Number of total samples by specimen type

Category	Clot	Urine	Serum + Plasma
Case	3347	4487	3445
Control	7511	10307	7929
	10858	**14794**	**11374**

Note: For each participant stored in the biological specimen bank, there are 2 clots, 3 urines, 1 serum, 1 heparin plasma, and 1 citrate plasma sample.

CRYOGENIC STORAGE SYSTEM DESCRIPTION

(Dr. Paola Muti and Frank Modlich)

LIQUID NITROGEN SYSTEM

The biological specimen bank consists of two tanks of liquid nitrogen and one freezer at -80°C. One of the tanks, marked with the number 1, stores the original part of the blood samples. The other tank, marked with the number 2, stores the duplicate of the blood samples. Since, in actuality, the samples are exactly the same (in terms of quantity and quality), the words "original" and "duplicate" are used as a convention, in order to organize the map of the bank. The freezer is marked with number 3.

Identifying Compartments Within the Liquid Nitrogen Tank:

The tank consists of four compartments which can be identified as follows: Compartment #1 is identified as the compartment which has a metal chain attached to the left side of the compartment wall. Moving counterclockwise, the next compartment is compartment #2, the next compartment is #3, and the last compartment is #4.

Each compartment consists of six rows of canisters which consist of the following colors from the outer circumference to the center (The color of the canister can be identified by a metal strip which has a plastic color tab on its end):

10 GREEN	**5 PURPLE**
8 RED	**4 GREY**
7 YELLOW	**2 PINK**

Each compartment consists of 36 canisters. Each of the canisters will be identified by a number and by its color. The numbers of the canisters will be sequentially assigned beginning with the first green canister in the left outside corner of the first compartment and going counter clockwise. For example: the first row consists of green canisters numbered one through ten. The second row consists of red canisters numbered forty-one through forty-eight. The third row consists of yellow canisters numbered seventy-three through seventy-nine. The fourth row consists of purple canisters numbered one hundred and one to one hundred and five. The fifth row consists of grey canisters numbered one hundred and twenty-one to one hundred and twenty-four. The sixth row consists of pink canisters numbered one hundred and thirty-seven to one hundred and thirty-eight.

Each canister consists of four goblets identified by color and number. The goblets will be arranged from bottom to top starting with **white** followed by **green** followed by **red** and finally **blue** on top.

Each goblet will be numbered from one to four, from the bottom to the top. The number will be written on the side of the goblet and on the lid.

Description of Location of Visotubes Within Each Goblet:

Each visotube contains one participant's samples. The visotubes are arranged counterclockwise by alphabetized color as follows:

1. **BLACK**
2. **BLUE**
3. **BROWN**
4. **GREEN** (lime green)
5. **GREY**
6. **LIGHT GREEN** (turquoise)
7. **ORANGE**
8. **PINK**
9. **PURPLE**
10. **RED**
11. **ROUND** (central visotube)

Procedure of Mapping

The specimens from each participant are divided equally into two visotubes of the same color. One is identified as the "**original**" (OR) and the other is identified as the "**duplicate**" (DUP).

The original and the duplicate visotubes are placed into separate goblets. These two goblets contain the specimens from the same participants, and their visotubes are arranged within the goblet following the established color order as described above. One goblet is considered to be the "original" and the other goblet is considered to be the "duplicate".

In the liquid nitrogen tank, compartment #1 is the first compartment which contains the goblets with the **ORIGINAL** visotubes. When the first row (bottom row) of compartment #1 is full, compartment #2 is used as the next compartment for the ORIGINAL visotubes. When the first row in both compartments is full, we begin to fill the second row of compartment #1 from the left outside corner to the right. Rows 3 and 4 follow the same pattern.

In the liquid nitrogen tank, compartment #3 is the first compartment which contains the goblets with the **DUPLICATE** visotubes. When the first row (bottom row) of compartment #3 is full, compartment #4 is used as the next compartment for the DUPLICATE visotubes.

When the first row in both compartments is full, we begin to fill the second row of compartment #3 from the left outside corner to the right. Rows 3 and 4 follow the same pattern.

The map of this specimen bank is kept in a record book in the laboratory supervised by Lyn Hill and Frank Modlich.

<u>**Liquid Nitrogen Storage Form**</u>

Original sample /__/ Duplicate /__/

Canister____________________/ /__________/ Goblet____________________//__________/
color number color number

ID_______________/ Visotube_______________/ Date of Storage in L.N. /___/___/___/

Time of Storage in L.N. /___:___/am/pm

ID_______________/ Visotube_______________/ Date of Storage in L.N. /___/___/___/

Time of Storage in L.N. /___:___/am/pm

ID_______________/ Visotube_______________/ Date of Storage in L.N. /___/___/___/

Time of Storage in L.N. /___:___/am/pm

ID_______________/ Visotube_______________/ Date of Storage in L.N. /___/___/___/

Time of Storage in L.N. /___:___/am/pm

ID_______________/ Visotube_______________/ Date of Storage in L.N. /___/___/___/

Time of Storage in L.N. /___:___/am/pm

ID_______________/ Visotube_______________/ Date of Storage in L.N. /___/___/___/

Time of Storage in L.N. /___:___/am/pm

Original sample /__/ Duplicate /__/

Canister_____________________/ /__________/ Goblet____________________//__________/
color number color number

ID______________/ Visotube______________/ Date of Storage in L.N. /___/___/___/

Time of Storage in L.N. /___:___/am/pm

ID______________/ Visotube______________/ Date of Storage in L.N. /___/___/___/

Time of Storage in L.N. /___:___/am/pm

ID______________/ Visotube______________/ Date of Storage in L.N. /___/___/___/

Time of Storage in L.N. /___:___/am/pm

ID______________/ Visotube______________/ Date of Storage in L.N. /___/___/___/

Time of Storage in L.N. /___:___/am/pm

ID______________/ Visotube______________/ Date of Storage in L.N. /___/___/___/

Time of Storage in L.N. /___:___/am/pm

ID______________/ Visotube______________/ Date of Storage in L.N. /___/___/___/

Time of Storage in L.N. /___:___/am/pm

Map of Storage Racks

As the storage boxes are filled, the order in which they will be placed into the freezer is as follows: The first complete box is placed in the last compartment of row 1, beginning on the left side. This box is number one; the following boxes are numbered sequentially. The number of the storage box should be written on the front of the box so that the first participant is oriented in the upper left corner. A number is also written on the lid of the box. Two numbers can be seen on the lid, one directly on top and the other on the side. The second box is placed on top of the first box and the next two boxes fill this compartment. The remaining compartments are filled following the order described above and filling from the back of the rack to the front. The racks are filled from the left to the right and from the bottom of the freezer (row 1) up to the top (row 5). Each rack has an assigned number following a sequential order.

-80°C Freezer Storage System Description

For this study, some of the specimens are stored in a -80°C freezer. These are extra samples which are stored after the straws have been filled for liquid nitrogen storage. These include the following: two blood clots, one serum sample, one sample of heparinized plasma, one sample of citrate plasma and three samples of urine. All samples are aliquoted into cryovials and placed into numbered storage boxes. This freezer is in the same room as the liquid nitrogen tanks.

The -80°C freezer used for storage of these samples is an upright Puffer Hubbard freezer. It consists of five horizontal shelves (rows). The rows are numbered as follows: The bottom row is designated as row number one. The second row going upward is row number two. The third row is number three. The fourth row is number four. The uppermost row is row number five. Each row contains five racks in which the storage boxes are placed. Each rack contains four compartments. Each compartment contains four storage boxes. Each contains eight rows and eight columns. Each box can hold a maximum of sixty four samples. Each horizontal row represents one participant.

Map of the Storage Box

Each storage box will be filled in the following order: The first participant's sample is placed in the box beginning with the upper left corner and filling to the right. The first two spaces in this row contain the two large vials used for the clots. These are followed by a serum vial, followed by a heparinized plasma vial, followed by a plasma citrate vial. The last three spaces contain three urine samples. Each vial is labeled with initials of the specimen sample (C=clot, S=serum, HP= heparinized plasma, CP= plasma citrate, U= urine) and the ID number of the participant.

The specimens from the next participant are placed in the second row beginning on the RIGHT SIDE of the box and filling to the left. The samples from the third participant will be placed in the third row beginning on the LEFT SIDE and filling to the right. The remaining rows are filled in an alternating pattern from the left to right and vice versa.

FREEZER MAP

RACK 17 ROW 5 BOXES 257-272	RACK 18 ROW 5 BOXES 273-288	RACK 19 ROW 5 BOXES 289-304	RACK 20 ROW 5 BOXES 305-320
RACK 13 ROW 4 BOXES 193-208	RACK 14 ROW 4 BOXES 209-224	RACK 15 ROW 4 BOXES 225-240	RACK 16 ROW 4 BOXES 241-256
RACK 9 ROW 3 BOXES 129-144	RACK 10 ROW 3 BOXES 145-160	RACK 11 ROW 3 BOXES 161-176	RACK 12 ROW 3 BOXES 177-192
RACK 5 ROW 2 BOXES 65-80	RACK 6 ROW 2 BOXES 81-96	RACK 7 ROW 2 BOXES 97-112	RACK 8 ROW 2 BOXES 113-128
RACK 1 ROW 1 BOXES 1-16	RACK 2 ROW 1 BOXES 17-32	RACK 3 ROW 1 BOXES 33-48	RACK 4 ROW 1 BOXES 49-64

COMPLETION OF BIOLOGICAL SPECIMEN BANK FORMS

Several measurements will be recorded prior to the blood draws each day. These will include the following: air temperature (to be recorded on each blood draw form), humidity, and atmospheric pressure (to be recorded in a separate record book in the blood drawing room). The individual responsible for these measurements will also perform the blood draws.

Blood Draw Form and Blood Collection Form

Before the bloods are drawn, the blood collection forms should be completed. The **blood draw form** is the first form to be completed. This form requires the following information: participant name, subject ID, date and time of the last meal, date of last period, time of last cigarette, history of bleeding problems, difficulty with blood draws, and time of last urination, along with additional information.

The **blood collection form** is the second form to be completed. On this form the following information is recorded: date, temperature of the room, label of the participant, and, just prior to the actual blood draw, the time.

After the bloods have been drawn, the number and type of vacutainers drawn are recorded on the **blood collection form**. Any problems which may arise during the blood draw procedure are recorded on this form. The individual responsible for performing the blood draw is also responsible for completing both of these forms.

Laboratory Form

When the blood samples are brought to the laboratory, the lab form needs to be completed. This form requires the following information: date, temperature of the lab, ID of technician and the ID label of each participant. After processing is completed and the straws are sealed, the number of actual straws filled is recorded on this form. In addition, the quantities of samples from both Dr. Armstrong and Dr. Barbarossa's lab are recorded. The last page of this form contains information regarding additional samples and tubes and extra specimens available. Information about volume and storage location is also included in this section.

IMPORTANT: The time at which the straws and vials are placed into the -80°C freezer must be recorded on the last line of this form.

Liquid Nitrogen Storage Form

After the straws have been sealed and placed into the visotubes, a corresponding form, **The liquid nitrogen tank: biological specimen lab report** needs to be completed. This form consists of two pages. One page is identified as the "original", and the other is identified as the "duplicate". It is necessary to check off which page is being completed. The second line on this form requires information regarding canister color, number and color/number of the goblet. Each page represents the contents of a half-filled goblet (six visotubes). Each of the pages (original and duplicate) needs to be placed into the large three ring record books. One book is identified as the "ORIGINAL", and the other as the "DUPLICATE".

IMPORTANT: The date and time at which the visotubes are placed into the liquid nitrogen tank are recorded after each participant ID line.

Every day, during the first month of activity, a technician reported on a form the level of the liquid nitrogen inside the tank, in order to estimate the quantity of liquid nitrogen needed for the bank every month.

Every day, during the entire study, a technician checked the outside temperature of the tank, by feel, to detect any leaks too small to be seen.

-80°C Freezer Storage Form

After the cryovials have been filled and closed, the corresponding yellow form needs to be completed. This form includes information regarding box number, position of the rack, and position of the rack in the freezer, indicating the actual position of the samples as they are stored in the storage box. For each participant, a label marked with an ID number is placed on the left side of the scheme.

Blood Collection
Blood Drawing Room

Code Number_____

Temperature of the room ___, __
(measure to the nearest decimal)

DATE___/___/___

Time of Blood Draw ___ ___:___ ___
HR MIN

IT IS VERY IMPORTANT TO FILL UP THE VACUTAINERS COMPLETELY.

1. (Mark the Filled Vacutainers)

Color	Yes	No	Possible Problem during blood draw:
1. GREEN Top			
2. RED Top			
3. LAVENDER Top (the small one)			
4. LAVENDER Top			
5. RED Top			
6. LIGHT BLUE Top			
7. URINE			

Laboratory Form

Code Number_____

Temperature of the room ___, __
(measure to the nearest decimal)

DATE____/____/____

I.D. Technician of LAB_____

Original Tube Color	Specimen	Straw color and (number)	Number of straws filled	*Problem
RED	Serum	Yellow (8)	☐	☐ P*
	Clot		☐ Y ☐ N	☐ P*
*Problem:				
GREEN	Plasma	White (6)	☐	☐ P*
	Buffy Coat	Blue (2)	☐	☐ P*
	Red Blood Cells	Green (4)	☐	☐ P*
1.5ml to Dr. Armstrong's lab			☐ Y ☐ N ☐ P*	
LIGHT BLUE	Plasma	Red (6)	☐	☐ P*
	Buffy Coat	Blue (2)	☐	☐ P*
*Problem:				

RED

2ml Vial Serum to Dr. Armstrong's lab ☐Y ☐N ☐P*

2ml vial Serum to Dr. Barbarossa's lab ☐Y ☐N ☐P*

CLOT ☐Y ☐N ☐P*

*Problem:_____

_____

Additional Samples and Tubes:

LAVENDER

10ml to Dr. Armstrong's lab ☐Y ☐N ☐P*

2.5ml to Dr. Barbarossa's lab ☐Y ☐N ☐P*

URINE ☐Y ☐N ☐P*

*Problem:_____

_____

EXTRA SPECIMENS AVAILABLE ☐Y ☐N

SERUM: __ __:__ __ml
HEPARIN PLASMA: __ __:__ __ml
CITRATE PLASMA:__ __:__ __ml

STORAGE BOX # _____

LINE #_____

LEFT☐ RIGHT☐ POSITION
TIME STRAWS FROZEN: __ __:__ __AM/PM

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Alcohol dehydrogenase 3 genotype modification of the association of alcohol consumption with breast cancer risk

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Abstract

Objectives: Because alcohol dehydrogenase 3 (ADH_3) is rate-limiting in alcohol oxidation and is polymorphic, we examined ADH_3 genotype in relation to alcohol intake and breast cancer risk.
Methods: We conducted a case–control study among Caucasian women aged 40–85 with incident, pathologically confirmed breast cancer and controls, frequency-matched on age and county. Queries included alcohol intake in the past 20 years. Genomic DNA was genotyped for the exon VIII ADH polymorphism by PCR followed by restriction enzyme digestion. Computation of odds ratios (OR) and 95% confidence intervals (CI) was by unconditional logistic regression.
Results: We found increased risk among pre- (OR 2.3, 95%, CI 1.2–4.3) but not postmenopausal women (OR 1.1, 95% CI 0.7–1.7) associated with $ADH_3^{1\text{-}1}$ compared to $ADH_3^{1\text{-}2}$ and $ADH_3^{2\text{-}2}$ genotypes. Risk was increased for premenopausal women with the $ADH_3^{1\text{-}1}$ genotype and alcohol intake above the median (OR 3.6, 95% CI 1.5–8.8) compared to lighter drinkers with the $ADH_3^{2\text{-}2}$ or $ADH_3^{1\text{-}2}$ genotypes. ORs were close to null for premenopausal women in other drinking and genotype groups and for postmenopausal women categorized by genotype and alcohol consumption.
Conclusion: Among premenopausal women there may be a group more genetically susceptible to an alcohol consumption effect on breast cancer risk.

Introduction

While there is evidence that alcohol consumption may increase the risk of breast cancer [1–3], the mechanism of action is not well understood. It is possible that genetic differences in the metabolism of alcohol may alter the relation of alcohol exposure to breast cancer. Evaluation of heterogeneous groups may mask susceptible subgroups and impair estimation of effects. In this study we evaluated genetic variation in alcohol dehydrogenase, a key enzyme in alcohol metabolism, as a modifying factor in the relation between alcohol intake and breast cancer risk.

Alcohol dehydrogenase (ADH) catalyzes the oxidation of ethanol to acetaldehyde and plays a rate-limiting role in the metabolic pathway for most human ethanol oxidation. Dimeric class I ADH enzymes are composed of subunits encoded by genes designated as ADH_1, ADH_2, and ADH_3. Genetic variants with altered kinetic properties have been identified at the ADH_2 and ADH_3 loci [4]. The aldehyde dehydrogenase family of enzymes (ALDH) is also involved in alcohol metabolism, and

variant alleles with altered kinetic activities have been identified in the $ALDH_2$ gene [4]. Polymorphisms in ADH_2 and $ALDH_2$ are rare in Caucasian populations [4–6]. In one study of the ADH_3 gene, approximately 58%, 91% and 88% of European whites, Asians and Africans, respectively, had the ADH_3^1 allele [5]. For this study of Caucasians we examined effects of the ADH_3 polymorphism. There is evidence that this variant has functional importance. *In vitro* there is more than two-fold difference in V_{max} between the ADH_3 genotypes [4], with the ADH_3^1 allele coding for the more rapid form of the enzyme.

While there are, to our knowledge, no reports on the association of ADH_3 in relation to breast cancer, there have been reports of an association of the $ADH_3^{1\text{-}1}$ genotype with increased risk of cancer of the oral cavity and pharynx [7, 8] and of hepatic cirrhosis and chronic pancreatitis [6]. We report here on the results of a case–control study of breast cancer risk with an examination of associations of alcohol consumption stratified by ADH_3 genotype.

Materials and methods

We conducted a case–control study of breast cancer in pre- and postmenopausal women in western New York State. All participants provided written informed consent; procedures for protection of human subjects in this study were approved by the Human Subjects Review Board of the State University of New York at Buffalo School of Medicine and Biomedical Sciences and of each of the participating hospitals. The women in the study were between the ages of 40 and 85, residents of Erie and Niagara counties, alert, able to speak English and in sufficiently good health to be interviewed; all were Caucasian. Women were considered to be premenopausal if they were currently menstruating, if they were not menstruating because of a hysterectomy or other medical intervention, or if they had at least one of their ovaries and were less than age 50. All other women were considered to be postmenopausal.

Women with incident, primary, histologically confirmed breast cancer were identified from pathology records of all the major hospitals in the two counties; case ascertainment was conducted in the period beginning November 1986 and ending October 1989 for postmenopausal cases, and ending April 1991 for premenopausal cases. The physician of each woman identified with breast cancer was contacted to obtain consent to allow us to invite the woman for an interview. Of eligible cases, 66% of premenopausal and 54% of postmenopausal cases were interviewed. Physician refusal to allow us to contact their patients accounted for most of the lack of participation, 74% and 71% of non-participation for pre- and postmenopausal women, respectively. Interviews were conducted, on average, 2 months after diagnosis; no interviews were conducted more than 1 year after diagnosis.

Controls were frequency-matched to cases on age and county. The listing of licensed New York State drivers was used for random selection of women under age 65; women age 65 and over were randomly selected from the listing of the Health Care Finance Administration. Sixty-two percent of the eligible premenopausal and 44% of eligible postmenopausal controls were interviewed. Because controls under age 65 were licensed drivers, we asked the cases under 65 if they had driver's licenses. Nine did not hold a driver's license. Compared to cases with licenses, women without licenses were slightly less educated and slightly, though not significantly, older. All are included in these analyses. For a subset of participating controls and those refusing to participate, we conducted a very brief phone interview querying usual frequency of consumption of several foods. These participants and non-participants did not differ in reported intake of vegetables, fruits, meat or coffee. Non-participants were somewhat more likely to smoke. Information was not collected on alcohol intake in this comparison of participants and non-participants [9, 10].

Interviews

Interviews were conducted in the participants' homes by trained interviewers. The interview lasted, on average, 2 hours. Details of the interview have been described elsewhere [9–11]. Included in the interview were questions regarding usual diet 2 years before the interview, reproductive history, medical history, family history of cancer, smoking history (pack-years) and other breast cancer risk factors. Body mass index (BMI) was calculated from reported height and weight, as weight (kg)/height^2 (m^2). Family history of breast cancer was defined as having at least one first-degree relative (mother, sister, daughter) with breast cancer.

Questions regarding alcohol intake included queries of the usual frequency of intake and number of drinks per occasion for wine, beer and hard liquor during the year 2 years ago, 10 years ago, 20 years ago and at age 16. Total alcohol intake was calculated as the sum of the reported number of drinks of beer, wine and hard liquor under the assumption that the alcohol content of one glass of beer or wine or one shot of hard liquor was approximately the same. An index of usual alcohol consumption in the last 20 years was estimated as a

weighted sum of the reported intakes for 2 years ago, 10 years ago and 20 years ago.

At the end of the interview, participants were asked to provide a blood sample following an additional informed consent. About 45% of premenopausal and 63% of postmenopausal participants agreed to give a blood sample.

Molecular genetic analyses

All analyses were conducted at the Laboratory for Human Carcinogenesis at the National Cancer Institute. DNA was extracted from blood clots [11]. As previously described [7], a 145 bp fragment including the Exon VIII polymorphism was amplified by the polymerase chain reaction (PCR) using a modification of the method of Groppi *et al.* [12]. The highly homologous ADH_1 and ADH_2 genes were digested with the NlaIII restriction enzyme prior to the PCR. An aliquot of this digestion mixture was then subjected to PCR and subsequent SspI enzymatic digestion to reveal the ADH_3 genotype (*i.e.*, ADH_3^{1-1}, ADH_3^{1-2}, or ADH_3^{2-2}). Every 14 samples contained a positive and negative control. The results were scored separately by two authors, independently, who were blinded to all identifying data including subjects' case–control status. Twenty percent of samples were repeated for quality control. In the adjusted analyses, NAT2 genotype was examined as a potential adjusting variable; methodology for the NAT2 analyses has been described previously [11].

The final sample for this report included 134 premenopausal cases and 126 premenopausal controls, 181 postmenopausal cases and 230 postmenopausal controls, those women whom we interviewed and whose ADH_3 genotype could be determined. Because we did not obtain blood samples from all participants who completed the interview, nor were we able to successfully determine the ADH_3 polymorphism on all blood samples, we compared the characteristics of those included in this report with the entire group included in the case–control study; comparisons of means were made using Student's *t*-test. Those with and without ADH_3 data were largely similar, with a few exceptions. Differences ($p < 0.05$) among premenopausal women were that those with data tended to be older, have higher parity and to drink less beer than those without. Among postmenopausal women, the only characteristic that was significantly different was age; those with ADH_3 data were older.

Statistical analysis

Because there are indications that there are differences in the risk factors for pre- and postmenopausal breast cancer [13], and in particular because there may be differences in the effect of alcohol intake depending on menopausal status [1], analyses were stratified by menopausal status. For potential confounding factors, means and standard deviations for groups defined by ADH_3 genotype and by case–control status were compared by one-way analysis of variance, with a two-tailed test of significance; values for categorical data were compared using the chi-square test [14]. Odds ratios (OR) and 95% confidence intervals (CI) were calculated using unconditional logistic regression [15]. For analyses of categorical data, ORs were calculated relative to the indicated referent category. Cutoffs for categories of alcohol intake were at the median level of intake for controls. Because of limitations in sample size it was not possible to examine groups with more narrowly defined alcohol intakes. Adjusted analyses included control for age, education, family history of breast cancer, reported history of benign breast disease, body mass index (BMI), parity, age at first birth, age at menarche, fruit and vegetable intake, duration of lactation and (for postmenopausal women) age at menopause. Most of these factors were examined for confounding effects because they have been found to be associated with risk of breast cancer. We also examined possible confounding by smoking history, NAT2 status and smoking by NAT2 interaction because we had previously found these to associated with risk in this population [11]. ORs for the ADH_3 genotypes were calculated and then ORs for alcohol intake both without and with stratification on ADH_3 genotype were calculated. Because of issues regarding differential recall for cases and controls in case–control studies, we also examined a case–case analysis in relation to alcohol dehydrogenase status; alcohol intake was regressed on ADH genotype among the cases with the ADH_3^{2-2} and ADH_3^{1-2} groups combined as the referent with comparison to ADH_3^{1-1} [16, 17].

Results

For all analyses the cutoff between the lower and higher groups of drinkers was at the median for controls, 6.5 and 4.4 drinks per month on average over the past 20 years, for the pre- and postmenopausal women, respectively. The associations between reported alcohol consumption in the past 20 years and risk of breast cancer in this sample of individuals with available genetic data are shown in Table 1. For both pre- and postmenopausal women, confidence intervals included the null. For the premenopausal women there was a suggestion of increased risk among heavier drinkers. Similar results

Table 1. Alcohol consumption in the past 20 years and risk of breast cancer, Western New York, 1987–91 (subgroup of women with alcohol dehydrogenase 3 (ADH_3) genotype measured)

Alcohol*	Cases	Controls	Crude OR	Adjusted OR†	95% CI†
Premenopausal					
Lower	54	63	1.0	1.0	
Higher	80	63	1.5	1.6	(0.9–2.6)
Total	134	126			
Postmenopausal					
Lower	93	113	1.0	1.0	
Higher	88	117	0.9	0.9	(0.6–1.5)
Total	181	230			

* Cutoffs for lower and higher groups of drinkers were the medians of the reported average consumption over the past 20 years; the cutoff was 6.5 and 4.4 drinks per month, for pre- and postmenopausal women, respectively.

† OR = odds ratio; CI = confidence interval. Adjusted for age, education, family history of breast cancer, history of benign breast disease, BMI, parity, age at first birth, age at menarche, fruit and vegetable intake, alcohol intake in the past 20 years, lifetime duration of lactation, and age at menopause (postmenopausal women only).

were obtained when all the data, including participants who did not provide a blood sample, were analyzed. We also examined risk associated with alcohol consumption separately for the reports of alcohol consumption 2 years ago, 10 years ago and 20 years ago. ORs for these periods were similar to those shown for the combined index; confidence intervals overlapped for all three periods for pre- and postmenopausal women.

In Table 2, breast cancer risk factors are shown for cases and controls grouped by genotype. In general, characteristics of the three genotype groups within the cases and the controls were similar. For alcohol intake, values shown are for all subjects combined, including non-drinkers. For the premenopausal women, 4% of cases and 6% of controls were non-drinkers; for postmenopausal women, non-drinkers constituted 13% of cases and 11% of controls. The percentage of non-drinkers did not differ by genotype in any of the groups defined by case–control and menopausal status. In one-way analysis of variance the reported alcohol intakes were not different by genotype for either the pre- or postmenopausal controls. For premenopausal cases with the ADH_3^{2-2} genotype, reported alcohol intakes were significantly higher than those with the ADH_3^{1-2} genotype ($p < 0.05$), but not the ADH_3^{1-1} genotype. There were also some differences in smoking history between the homozygotes and the heterozygotes among the premenopausal women. Among postmenopausal women, alcohol consumption and smoking did not differ for the different groups; there was a difference by genotype for education among the cases.

In Table 3, risk of breast cancer associated with ADH_3 genotype is shown. There was an increase in risk for the premenopausal women associated with the ADH_3^{1-1} genotype; the confidence interval included the null value (adjusted OR 2.0, 95% CI 0.8–4.6). There was little evidence of an association of genotype with risk for postmenopausal women. ORs estimated without adjusting for alcohol intake were similar to those shown here. Addition of smoking, NAT2 and an interaction term of NAT2 and smoking did not appreciably change the estimates.

We also examined risk of breast cancer associated with the ADH_3^{1-1} genotype when the referent was the ADH_3^{2-2} and ADH_3^{1-2} genotype groups combined. For premenopausal women the OR was 2.3 (95% CI 1.2–4.3); for postmenopausal women the OR was 1.1 (95% CI 0.7–1.7) (data not shown).

In Table 4, ORs for alcohol intake by ADH_3 genotype are shown. The referent was women with lower intake of alcohol and either the ADH_3^{2-2} or ADH_3^{1-2} genotype. (We also analyzed these data with ADH_3^{2-2} alone as the referent. The results were similar to those shown here. However, the findings were less stable because the sample size in the reference group was small and CI were wider.) Among the premenopausal women, ORs were generally close to the null and CIs included the null for all categories with one exception. Among women who drank more than the median intake and who had the ADH_3^{1-1} genotype, the OR was 3.6 with 95% CI 1.5–8.8. It appeared that the effect associated with both the ADH_3^{1-1} genotype and higher alcohol consumption was more than additive; however, the multiplicative interaction term in a logistic regression was not significantly different from the null ($p = 0.16$). The estimates of risk in Table 4 were essentially unchanged when smoking, NAT2 and smoking × NAT2 were included in the model. We also examined risk associated with alcohol within the group of women with the ADH_3^{1-1} genotype. With lighter drinkers as the referent, the adjusted OR for drinking more than the median of alcohol was 3.9, 95% CI 1.3–10.1 (data not shown). Additionally, we repeated this latter analysis, changing the cutpoint for the low and high drinkers so that there was an even distribution within the premenopausal controls with the ADH_3^{1-1} genotype. The results were essentially the same (OR 3.9, 95% CI 1.4–10.9).

Among postmenopausal women there was no evidence of an association of alcohol intake and risk when modification by ADH_3 was taken into account. Because of reports that an increased risk associated with alcohol consumption among postmenopausal women may be restricted to those who have used estrogen replacement therapy (ERT) [18, 19], we also looked at the OR among

Table 2. Characteristics of study sample by case and control status and alcohol dehydrogenase 3 (ADH_3) genotype

Characteristic*	Cases			Controls		
	ADH_3^{1-1}	ADH_3^{1-2}	ADH_3^{2-2}	ADH_3^{1-1}	ADH_3^{1-2}	ADH_3^{2-2}
Premenopausal women						
Age (years)	46.2 (4.6)	46.8 (4.0)	45.0 (3.2)	46.2 (3.2)	46.9 (3.7)	47.5 (4.5)
Education (years)	13.6 (2.5)	14.0 (3.2)	14.1 (2.5)	14.1 (2.4)	13.6 (2.8)	14.2 (2.6)
Age at menarche (years)	12.6 (1.8)	12.4 (1.6)	12.5 (1.2)	13.1 (1.9)	12.9 (1.7)	13.3 (1.7)
Body mass index†	24.2 (5.2)	25.2 (6.0)	24.7 (4.8)	25.2 (4.6)	25.6 (4.1)	26.6 (6.0)
History of benign breast disease (percentage of cases or controls)	22	20	7	15	13	9
Family history of breast cancer (percentage of cases or controls)	8[a]	8[b]	2[a,b]	0.8	0.4	0.8
Total alcohol† (drinks/month)	16.8 (20.2)	9.7[c] (11.3)	20.2[c] (24.4)	14.2 (28.0)	13.4 (18.1)	12.5 (12.8)
Duration lactation (months)	4.0 (8.6)	2.1 (4.3)	2.7 (5.0)	8.0 (14.1)	5.2 (12.1)	6.2 (13.1)
Parity	2.5 (1.6)	2.1 (1.5)	1.9 (1.3)	2.4 (1.6)	2.8 (1.8)	3.0 (1.7)
Age at first birth (years)	24.0 (4.4)	24.0 (4.8)	23.8 (5.2)	22.8 (4.0)	21.9 (4.1)	21.8 (4.0)
Vegetable intake† (g/day)	459 (220)	395 (180)	419 (175)	462 (190)	473 (201)	450 (155)
Fruit intake† (g/day)	239 (133)	210 (141)	170 (125)	272 (170)	245 (149)	216 (112)
Smoking (pack-years)	11.7[d] (16.4)	5.8[d] (10.1)	12.6 (14.4)	5.7[e] (11.4)	11.7[e,f] (16.6)	4.8[f] (8.9)
n	63	50	21	42	60	24
Postmenopausal women						
Age (years)	64.9 (6.4)	63.6 (7.8)	61.9 (7.5)	63.4 (7.7)	63.1 (7.2)	61.6 (6.7)
Education (years)	12.2[e] (2.6)	12.3 (2.9)	13.4[e] (3.2)	12.3 (2.6)	12.0 (2.3)	12.7 (2.5)
Age at menarche (years)	13.0 (1.8)	13.0 (1.6)	12.6 (1.4)	12.7 (1.7)	13.1 (1.6)	12.6 (1.3)
Age at menopause (years)	47.8 (5.3)	47.6 (6.1)	46.8 (5.5)	46.2 (6.0)	47.6 (5.3)	47.0 (6.0)
Body mass index†	25.7 (5.3)	26.0 (5.0)	25.6 (3.6)	25.2 (4.2)	25.7 (5.4)	25.4 (4.7)
History of benign breast disease (percentage of cases or controls)	6	12	2	8	8	3
Family history of breast cancer (percentage of cases or controls)	6	6	5	3	6	1
Total alcohol† (drinks/month)	11.8 (21.9)	17.1 (31.4)	17.7 (29.8)	10.6 (16.6)	15.9 (25.2)	12.6 (15.2)
Duration of lactation (months)	3.5 (5.7)	4.6 (11.0)	4.0 (8.0)	6.2 (10.7)	4.2 (9.0)	5.1 (10.0)
ERT (percentage ever used of cases or controls)	9	11	6	10	15	9
Parity	3.1 (2.0)	2.7 (2.0)	3.2 (2.8)	2.8 (2.2)	3.1 (2.0)	2.9 (1.8)
Age at first birth (years)	24.8 (5.0)	24.1 (4.9)	23.4 (5.2)	23.3 (4.6)	23.5 (4.6)	23.3 (3.8)
Vegetable intake† (g/day)	451 (201)	406 (175)	417 (207)	458 (237)	456 (227)	484 (334)
Fruit intake† (g/day)	298 (175)	254 (175)	287 (177)	306 (186)	282 (172)	308 (218)
Smoking (pack-years)	14.6 (21.0)	17.4 (21.3)	16.9 (29.1)	12.9 (16.5)	13.8 (19.3)	13.0 (23.0)
n	64	89	28	81	114	35

* Values shown are mean (SD) except for history of benign breast disease and family history of breast cancer which are percentages with positive history. Two-sided comparisons of means between the ADH_3 groups within cases or controls were computed by ANOVA; comparisons of categories were with the chi-square test. Those with the same letter are significantly different, $p < 0.05$.

† Body mass index (kg/m^2) calculated from reported height and weight 2 years before the interview. Alcohol values are average drinks per month during the past 20 years, calculated from the weighted sum of reported consumption 2, 10 and 20 years ago; values include non-drinkers. Vegetable and fruit intake is reported intake in the year 2 years before the interview.

women who had ever used ERT. Among the heavier drinkers with the ADH_3^{1-1} genotype compared to lighter drinkers with the other ADH_3 genotypes for women who had ever used ERT the adjusted OR was 1.2 and the 95% CI 0.8–1.7; for those who had never used ERT the OR was 1.0 and the 95% CI 0.9–1.6. Sample size was quite small for the cells in these analyses; there were only 10 cases and 9 controls with the ADH_3^{1-1} genotype that had ever used ERT. All of these analyses were based on reports of alcohol consumption in the past 20 years. We had also queried regarding alcohol intake at age 16. The number of drinkers at that age was too small to estimate whether there was a modifying effect of ADH_3 genotype.

In a case–case analysis we examined risk associated with the ADH_3^{1-1} genotype compared to the combined ADH_3^{1-2} and ADH_3^{2-2} groups. As for the case–control analyses, there was evidence of some increase in risk

Table 3. Alcohol dehydrogenase 3 polymorphisms and risk of breast cancer, Western New York, 1987–91

ADH_3	Cases	Controls	Crude OR	Adjusted OR*	95% CI*
Premenopausal					
2-2	21	24	1.0	1.0	
1-2	50	60	1.0	0.8	(0.4–1.8)
1-1	63	42	1.7	2.0	(0.8–4.6)
Total	134	126			
Postmenopausal					
2-2	28	35	1.0	1.0	
1-2	89	114	1.0	1.1	(0.6–2.1)
1-1	64	81	1.0	1.2	(0.6–2.3)
Total	181	230			

* OR = odds ratio; CI = confidence interval. Adjusted for age, education, alcohol intake, family history of breast cancer, history of benign breast disease, BMI, parity, age at first birth, age at menarche, fruit and vegetable intake, lifetime duration of lactation and age at menopause (postmenopausal women only).

Table 4. Lifetime alcohol consumption by ADH_3 genotype and risk of breast cancer, Western New York, 1987–91

Alcohol*	Cases	Controls	Crude OR	Adjusted OR†	95% CI†
Premenopausal					
ADH_3^{2-2} + ADH_3^{1-2}					
Lower	33	38	1.0	1.0	
Higher	38	46	1.0	0.8	(0.4–1.7)
ADH_3^{1-1}					
Lower	21	25	1.0	1.0	(0.4–2.5)
Higher	42	17	2.8	3.6	(1.5–8.8)
Postmenopausal					
ADH_3^{2-2} + ADH_3^{1-2}					
Lower	60	69	1.0	1.0	
Higher	57	80	0.8	0.8	(0.5–1.4)
ADH_3^{1-1}					
Lower	34	46	0.8	0.9	(0.5–1.6)
Higher	30	35	1.0	1.2	(1.1–2.2)

* Cutoffs for lower and higher groups of drinkers were the medians of the reported average consumption over the past 20 years; the cutoff was 6.5 and 4.4 drinks per month, for pre- and postmenopausal women, respectively.

† OR = odds ratio; CI = confidence interval. Adjusted for age, education, family history of breast cancer, history of benign breast disease, BMI, parity, age at first birth, age at menarche, fruit and vegetable intake, duration of lactation and age at menopause (postmenopausal women only).

associated with the ADH_3^{1-1} genotype for pre- but not postmenopausal women. For premenopausal women, risk was more than doubled for women drinking more than the median compared to lighter drinkers (adjusted OR 2.5, 95% CI 1.1–8.4). For the postmenopausal women the adjusted OR was 1.0 and the 95% CI was 0.4–2.1 (data not shown).

Discussion

This study of women in western New York provides evidence that the association of alcohol consumption with breast cancer risk may differ depending on genotype. Among premenopausal women, we found an increase in risk of more than 3.5-fold for drinkers above the median with the ADH_3^{1-1} genotype. We did not find an increase in risk for heavier drinkers with the other genotypes. Further, we did not find any indication in this population of generally light drinkers of a modifying effect of ADH_3 genotype among postmenopausal women. To our knowledge this is the first study of the relation of the ADH_3 polymorphism with alcohol and breast cancer risk. As noted above, there is some indication of an increase in risk of other alcohol-related diseases among individuals with the ADH_3^{1-1} genotype, including reports of a 2.5–6-fold increase in risk of oral and pharyngeal cancer [7, 8]. There are a considerable number of studies that indicate that alcohol is related to increased risk of breast cancer [1–3]. Some [20–24], but not all [1] studies find risk associated with alcohol intake particularly among premenopausal women.

This modification of the association between alcohol consumption and risk of breast cancer by ADH_3 genotype may provide some indication as to the mechanism of effect of alcohol exposure. Alcohol metabolism in humans is regulated primarily by the ADH system of enzymes. There is considerable evidence that acetaldehyde, the product of alcohol dehydrogenase oxidation of alcohol, has carcinogenic properties [25]. Acetaldehyde is mutagenic and carcinogenic in experimental animals. In short-term cell culture assays, including assays of human cells, acetaldehyde but not ethanol is mutagenic [26, 27]. *In vitro*, acetaldehyde effects include DNA adducts [28, 29], DNA crosslinks and DNA–protein crosslinks [30, 31] and inhibition of DNA repair [30]. The International Agency for Research on Cancer (IARC) has indicated that the evidence regarding acetaldehyde is sufficient for it to be designated as a carcinogen in experimental animals [32]. *In vitro* the V_{max} for ADH_3^{1-1} is more than 2-fold greater than for ADH_3^{2-2} [4], and may therefore contribute to increased exposure to acetaldehyde. It should be noted, however, that in one study in Caucasians no difference was found in blood ethanol levels for different ADH_3 genotypes [33]. There is evidence of measurable levels of circulating acetaldehyde in pre-

menopausal women after consumption of moderate amounts of alcohol during the high estrogen phases of the menstrual cycle [34, 35]. There is also evidence of acetaldehyde excretion in human milk [36]; however, the determinations in milk were not made in conjunction with alcohol consumption. ADH_3 expression is greatest in the liver; however, there is evidence of ADH_3 activity in other organs [37–42] with an indication of expression, particularly in epithelial cells [41].

Another possible mechanism involving ADH and alcohol is with regard to steroid hormone metabolism. There is strong evidence that estrogen exposure is an important contributor to breast cancer risk [43]. Alcohol consumption appears to affect estrogen levels; there is evidence that both acute [44–46] and chronic [47–49] alcohol consumption lead to increased estrogen levels in premenopausal women and in postmenopausal women who take exogenous estrogen. ADH_3 is also involved in steroid hormone metabolism and is inhibited by testosterone [50, 51]. If the association of ADH_3 with risk is the result of an interaction with steroid hormones, that mechanism might explain why we saw an association with risk only among the premenopausal women. Given the toxic effects of acetaldehyde, the apparent likelihood of exposure to breast tissue of acetaldehyde and the interactions of alcohol, ADH_3 and estrogens, these mechanisms together may explain, at least in part, an association of alcohol consumption with breast cancer risk. Of course, there are other possible mechanisms that may also explain the association of alcohol with breast cancer risk, which also need to be considered.

In interpretation of these findings, several potential sources of error need to be considered. In this study, all measures of alcohol intake were by self-report, and measurement error is of concern. However, there is some evidence that reliability of recall of intake of alcohol in the past 5–10 years is relatively good [52, 53], although current drinking practices may bias recall of intake [52]. In data such as ours, there is also the concern of recall bias, that women with recently diagnosed breast cancer may report their previous alcohol intake differently than the healthy controls do. In one study, this potential source of bias accounted for only a small reduction in the relative risk estimate with bias toward the null [54]. As for the measure of ADH_3 status, there may also be some misclassification of the clinically significant ethanol oxidation phenotype. Methodologically, however, laboratory personnel were blinded to case–control status; error with regard to ADH_3 status would be non-differential and would contribute to an attenuation of the odds ratio estimate [55].

In terms of the selection of the sample, while every effort was made to include a population-based sample in this study, there were several sources of non-participation. For the cases, the largest source of non-participation was the refusal of physicians to allow us to contact the women. It may be that this lack of inclusion reflects physician rather than patient characteristics, but we could not verify whether or not this was true. Among the controls we do have some evidence that, at least for dietary intake, there were no differences among participants and those who did not participate [9, 10]. There may have been differences in alcohol intake of those refusing to participate; in particular it is possible that the heaviest drinkers in the population were underrepresented. For both cases and controls there is no reason to believe that participation would be related to ADH_3 polymorphism; the frequency of the ADH_3^1 and ADH_3^2 alleles measured in this population (59% and 41%, respectively, among the controls) were similar to those reported by others [4, 5, 56]. ADH_3 would be unlikely to affect alcohol consumption; studies of ADH_3 in Caucasians have not shown there to be differences in risk of alcoholism associated with the ADH_3 genotype [6, 56]. We did not find any difference in alcohol intake by ADH_3 polymorphism among the controls. Among premenopausal cases reported alcohol intake was lower for the $ADH_3^{1\text{-}2}$ genotype than for the $ADH_3^{2\text{-}2}$; this finding does not make biological sense in the context of the other groups where there were no differences. It may be that this apparent difference is the result of chance. There were no differences in intake for the other comparisons within the cases, for the controls or for the postmenopausal cases or controls.

Possible confounding is also of concern. Known risk factors for breast cancer were examined as potential confounders. The possibility remains that there were other correlated exposures that may explain the observed associations. In particular, it could be that there is confounding by genetic admixture even within this group of Caucasians. In addition, rather than the observed associations being an effect of ADH_3, it could be that the observed association is the result of linkage disequilibrium of ADH_3 with another gene causally related to breast cancer.

Finally, there is also the possibility that these findings were the result of chance. Given the small samples in some of the cells of analysis, and given the issues of potential bias, these results necessarily need to be considered as preliminary and await confirmation by other, larger epidemiologic studies. Because of the restriction by sample size we were only able to categorize participants into two levels of drinking. The group of heavier drinkers necessarily included women whose

alcohol consumption was in fact rather low. Additionally, the group of lighter drinkers included both non-drinkers and those who drink less frequently. With larger sample size and the ability to examine risk in groups that are more narrowly defined by alcohol consumption, it would have been possible to elucidate the association of drinking, genotype and risk.

Our data suggest that genetic differences in alcohol metabolism by ADH_3 should be considered as possible modifiers of the association between alcohol intake and breast cancer. In other studies, consideration of genetic variation in ADH_2 and ALDH, which we were not able to study, is warranted. Further, given the small number of individuals in some of the genotype–alcohol categories, chance may explain the findings; replication in other populations would be of importance. Our findings of an apparent modification of effect by ADH_3 genotype, if confirmed in other studies, would shed some light on the possible mechanism of an alcohol consumption effect on breast cancer and indicate a high-risk group for an alcohol effect.

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Why and how to use a biological specimen bank in epidemiological and clinical research: Methodological issues

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Abstract

The use of biological specimen banks has proliferated in the last decade of scientific research. The present paper analyzes some issues that need to be taken into consideration by those researchers who carry out studies using biochemical determination as a measure, for example, of exposure and disease definition. Relevant considerations regarding specimen banking at very low temperatures (lower than -70°C) for a long time (years) include conditions and temperature of storage. Cryogenic containers (liquid nitrogen tanks and mechanical freezers) are described in terms of their potential advantage and disadvantage for biological specimen bank long term storage, freezing conditions (slow and fast freezing) and storage units are also described in terms of suitability of their use in specimen banks. One of the main aims of a biological specimen bank is to preserve specimens for future determinations to test hypotheses which at the time of the specimen bank establishment are not yet conceived or for which there are not suitable laboratory techniques. As a consequence, special attention should be paid by researchers to some important issues when designing a specimen bank. The choice of what to store, where to store, and how to store has a critical influence on the development and future scientific use of a biological specimen bank.
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Introduction

Biological markers from samples of blood, urine, or cells collected during epidemiological and clinical studies and stored for long periods can yield important information on: 1) the exposure of a study participant to endogenous and exogenous factors (e.g., hormonal or metabolic patterns and substances bound to serum, cellular protein or DNA); 2) incipient disease in the study participant at the time of the baseline examination; and 3) individual susceptibility to disease (e.g., gene isoform expression). Biological specimen banks (BSBs) have been and are likely to remain of great value in case-control, cross-sectional and cohort studies. Their use has proliferated in the last decade of epidemiological and clinical research largely because of progress in analytical and laboratory methods (1). For instance, new techniques allow better analysis of the complex interaction between genetic markers and environmental exposures. Furthermore, the development of micromethods and new biochemical and molecular laboratory techniques allow identification of complex biological markers in large study samples and thus optimize the cost-effectiveness ratio. This paper examines some issues that need to be taken into consideration in the establishment of a BSB.

Cryogenic containers

There is general consensus that the safest method to store biological samples for long-term storage is to freeze and store them at very low temperatures (-70°C and below) (2), where most proteins, steroids and vitamins remain stable. Some BSBs use liquid nitrogen tanks for storing their biological samples (1, 3), others use mechanical freezers (4, 5).

Liquid nitrogen tanks are steel containers which provide thermal isolation by means of a vacuum sealed space. They may hold thousands of aliquots and can be used either in

liquid or in gas phase. In liquid phase, the stored biological material is completely immersed into the liquid nitrogen at -196°C. In gas phase, liquid nitrogen level is kept at usually 10 cm from the bottom of the container and most of the biological material is exposed only to the cold vapor generated by the liquid nitrogen on the bottom.

Mechanical freezers store biological material at temperatures ranging from -70°C to -135°C, depending on the power of their compressor. They are sometimes constructed with two independent compressors to efficiently maintain temperature and provide protection in the event of the mechanical failure of one. Mechanical freezers may be upright or horizontal.

There are several advantages and disadvantages connected with the use of either liquid nitrogen tanks or mechanical freezers. One of the advantages of liquid nitrogen is that biological materials can be frozen at temperatures much lower than previously possible (-196°C *vs* -135°C and -70°C/-80°C in mechanical freezers). We know, in fact, that a number of biological parameters is affected by long-term storage at -80°C. Serum and plasma progesterone concentrations, for example, decrease, whereas serum free testosterone concentration increases during a three-year storage (6). Similar decay over time with storage at -70°C has been noticed for apolipoproteins (2) and polyunsaturated fatty acids (7). Long-term stability of the very unstable blood group antigens and red cell enzymes (glucose-6-phosphate and 6-phosphogluconate) have been observed for storage at -196°C (8-10). Though more studies are clearly needed, these findings suggest a promising area for reducing the loss of potentially valuable information over time by using liquid nitrogen.

Another advantage of using liquid nitrogen in the liquid phase is that it allows specimens to be stored in a liquid atmosphere at uniform temperature (-196°C) throughout the specimen bank. In contrast, in liquid nitrogen tanks in the gas phase, and sometimes in mechanical freezers, the temperature may vary (11). For instance, in the ORDET study (an Italian prospective study on hormones and diet in the aetiology of breast cancer provided with a BSB), a difference of 20°C between the top and the bottom of the BSB -80°C chest freezers was observed (P. Muti, unpublished data). The effect of different long-term storage temperature should be monitored and controlled in data analysis. Given the uniformity of temperature of liquid nitrogen in the liquid phase, specimens stored by any other method must therefore be analyzed to determine the possible effect of variations in temperature during storage. This weakens the power of the study because of the misclassification induced by potential effects of differences in storage temperature, and because of the consequent data matching and/or stratification by temperature levels.

A third advantage is that BSBs in liquid nitrogen do not depend on electrical power. In the event of a general blackout, the nitrogen level can be manually controlled and liquid nitrogen can be added. For this reason, a manual liquid nitrogen filling system should always be added to the automatic system usually installed and sold with the tanks. One of the problems in storing samples in a BSB in liquid nitrogen is the possibility of cracking of the liquid nitrogen tank due to the loss of vacuum between the tank walls. This can occur due to improperly welded joints or by aging of the tanks. An additional problem is the cost of liquid nitrogen.

There are advantages to the use of mechanical freezers. Freezers are more accessible for storing and recovering the samples: vapor from the liquid nitrogen makes it very difficult to see and retrieve samples stored in a tank. Furthermore, mechanical freezers need fewer special safety measures, such as masks for lab technicians, and sensors to monitor the oxygen concentration in the room. Liquid nitrogen tanks and mechanical freezers require the use of insulated gloves. The disadvantage of using mechanical freezers is their complete dependence on both electric power and the mechanical compressor. In case of a major power outage or compressor failure, CO_2 (or liquid nitrogen) cylinders connected to the freezers as an emergency cooling system can maintain the very low internal temperature for a short period before they need to be changed. Changing itself is a complex process that must be programmed through a detailed emergency protocol. Furthermore, trained personnel have to be constantly available and contacted via a phone alarm system. Compressor failure can become more frequent as freezers age. Among the costs for long-term storage in mechanical freezers, it is important to consider the expense of labor and replacement parts to maintain freezers, or the purchase of new freezers.

The risk of contamination is a possible source of problems in liquid nitrogen storage in liquid phase that does not exist with freezers and nitrogen in gas phase. It is not common when a BSB begins to operate, but can arise from microorganisms, viruses and oxygen. Contamination can be due to interaction between the contaminated liquid nitrogen and the biological contents due to defective cleaning of tanks, canisters, gloves. Liquid nitrogen has no sterilizing properties and there are two main methods to check for bacterial contamination of a tank: a) immersion of sterilized cotton into liquid nitrogen for few seconds and, after evaporation,

and liquid nitrogen tanks. However, they are expensive and not easy to fill. They can be stored in a small plastic unit holding 10 or 20 straws, depending on their volume (0.5 and 0.25 ml respectively). Each unit can be placed in a bigger container (plastic glass, aluminum drawers). When the straws are filled, they are ready to be frozen for long-term storage in both liquid nitrogen tanks and freezers at very low temperature.

Cryogenic vials range from 0.25 to 50 ml in volume. They are less expensive than straws and their filling usually requires less sophisticated equipment. They are made of polypropylene, polystyrene, PVC, silicone rubber, teflon or borosilicate glass. Silicone, teflon and PVC may affect serum during storage (16) due to absorption of less polar steroids (*ie*, progesterone and androstenedione) by those commonly used for laboratory plastic tubing. Vials for long-term storage should have a threaded plug and a hermetic seal to reduce the risk of dehydration and oxidation during storage. Moreover, if the specimens are stored in liquid nitrogen, the hermetic seal protects liquid nitrogen from seeping into the vials and the risk of vial breakage during thawing. Vials are usually stored in small boxes with partitions.

Labeling of straws and vials

Straws and vials can be labelled for long-term storage in several ways. A plastic jacket provided by the manufacturers can be wrapped around a straw to label it in liquid nitrogen tanks and mechanical freezers. The number for each stored sample (for instance, the identification number for each subject participating in the study) is printed on this label. Since specimens such as serum and plasma cannot be distinguished when frozen, the labels can be of different colours, e.g., serum: yellow label; plasma: red label; buffy coat: blue label; red blood cells: green label. For sample identification on vials, the use of computer-generated labels should be considered to avoid possible transcription errors: the normal ink of the old computer printers and the paper labels used in frozen food packaging seem to be adequate for labelling vials to be stored at -80°C for a long time.

Bar code labels are available for straws and vials. Their use can hasten laboratory and bank mapping procedures, though their automatic reading on frozen straws or vials may be difficult.

Conclusion

Several considerations are important in designing a BSB. The type of specimen to be stored (*ie*, serum, plasma, saliva, urine, or cells) and the mode of collection (e.g., fasting state, overnight urine collection, urine morning spot) are imposed by the scientific hypotheses supporting a BSB and the practical organization of a given study. For instance, an investigation on glucose metabolism will require blood withdrawals in the fasting state, whereas for urine storage the biomarker to be studied will determine whether morning spot urine, 12 h or overnight collection is required (17).

The methods of storage depend in general upon all the factors mentioned above and on budget considerations. Liquid nitrogen storage is in general more expensive than storage in mechanical freezers. The quantity of specimen for each aliquot, a decision that in turn dictates freezing space needs, should not be larger than the amount necessary for biochemical determinations related to the hypothesis to be investigated. Small aliquots can be used in different laboratories or at different times, whereas large aliquots may easily be wasted because of problems associated with thawing and freezing (18).

Furthermore, BSB storage raises some specific methodological issues. Aliquot preparations must be simple and highly standardized to ensure the quality and integrity of the specimens. Preservatives and additives must be carefully chosen, since they limit certain types of determinations (e.g., EDTA *vs* heparin *vs* citrate), and straws and vials must be chosen carefully to avoid unwanted chemical interactions between their constituents and the specimen. Lastly, when designing a BSB, the long-term effect of freezing and storage on the specimen should be considered. Several studies have documented decreases and/or increases in the concentrations of several hormonal and nutritional parameters of frozen specimens, even those stored at very low temperatures (6, 19, 20). It is thus important to take into account the specific long-term effects of freezing on the biological parameters of interest when designing a BSB.

The design of a BSB is fundamental to the use of biological markers in epidemiological and clinical studies. BSBs have made possible a new generation of studies that promise to illuminate unexplored dimensions of long-existing research (20). Diseases with a major impact on the population, such as certain chronic degenerative diseases, but whose incidence is insufficiently frequent to allow relatively fast analysis can be more readily studied through comprehensive and complex risk models that, in turn, will have been based on and made possible by BSBs.

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WHY AND HOW IN PREVENTION
AND CLINICAL CARE

Why and how to measure urinary sex steroid metabolites in epidemiological studies in women

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Abstract

Background and Aim: *Although numerous investigations have evaluated the association between urinary hormone levels and chronic diseases such as breast cancer and coronary heart disease, there are few data about the reliability of urinary measurements, particularly among premenopausal women.*
Methods and Results: *Over a six-month period, levels of estrone-3-glucuronide and pregnandiol-3-glucuronide were measured in both morning spot and overnight urine samples from seven healthy premenopausal women (ages 33-46). During this period, each subject provided one morning spot urine sample and one overnight urine sample per menstrual cycle on the same day of her menstrual cycle.*

All these samples were taken out of the freezer simultaneously and sent in the same parcel on dry ice to the laboratory for hormone determinations. All samples from each person were assayed simultaneously in the same run and by the same laboratory technician in a blind fashion. The intraclass correlation coefficients (ICC) for estrone-3-glucuronide and pregnandiol-3-glucuronide for the morning spot and overnight urine samples were 0.78 and 0.46 and 0.75 and 0.64 respectively.
Conclusions: *These data suggest that morning spot urine determinations are reliable and constitute an efficient alternative to the more complex overnight urine collection for epidemiological evaluation of urinary hormonal profiles.*
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Introduction

The usefulness of measurement in clinical and epidemiological research depends on the extent to which a researcher can rely on data as accurate and meaningful indicators of an exposure or presence of disease. Thus, reliability or the extent to which a measurement is consistent and free from error is a very significant aspect that needs to be addressed in the planning of research protocols. Reliability can be conceptualized as predictability: for instance, a reliable instrument is one that will perform with predictable consistency under specific conditions.

Theoretically, any "observed score" (X) can be viewed as a function of two components: a "true score" (T) and an "error component" (E).

In analytical determinations of biomarkers, E has two sources: technical variability (related to the act of measuring), and biological variability (due to the biological characteristics of a specific biomarker, e.g., circadian variation of several serum biomarkers).

This paper describes "why and how" it is important, in planning clinical and epidemiological research on hormone related disease, to consider E due to hormone biological variability, and illustrates the effect of this error in studies including estrone-3-glucuronide and pregnandiol-3-glucuronide determination in urine.

Many epidemiological studies have investigated the association between endogenous sex steroid levels and chronic diseases such as breast cancer and coronary heart disease (1-3). Serum hormone levels are thought to be the most accurate indicators of secretion patterns, whereas urinary levels reflect the end product of a complex path of secretion, metabolism and excretion (4). Inconsistent results have been found in case-control studies using urinary estrogens to determine the endocrine patterns associated with

the risk of breast cancer (5-15). These studies often relied on a single measurement of urinary hormone level, although there was limited information about the reliability of these parameters, particularly for premenopausal women. Large intra-individual variability may lead to misclassification of subjects and attenuated risk ratios (16-18). The present study was conducted to evaluate the reliability of measurements of two urinary glucosiduronate metabolites of serum steroids: estrone-3-glucuronide, and pregnandiol-3-glucuronide in morning spot and overnight urine samples.

Estrone-3-glucuronide is one of the main products of serum estrogen inactivation and conjugation, and provides a useful measure of ovarian function (19,20). Furthermore, it correlates with serum level of serum estradiol, the main serum estrogen in premenopausal women (4). Pregnandiol-3-glucuronide is the main product of serum progesterone. Its urine values are highly correlated with serum progesterone levels, which makes it an important biomarker of ovarian luteal function (21). In the present study, healthy premenopausal women provided monthly urine samples during a six-month period.

The subjects were in different phases of the menstrual cycle, but each woman provided urine samples for the same day of her menstrual cycle across the six months. The study design checked for several potential sources of biological (*ie*, circadian rhythm) and laboratory variability.

Methods

Study methods

This study was a pilot investigation for a series of case-control studies of lifetime alcohol intake and chronic disease among persons living in Erie and Niagara Counties in New York state. The protocol was approved by the Internal Review Board of the State University of New York at Buffalo. Participants were seven premenopausal women aged 33 to 46, non-smokers, in generally good health and with regular menstrual cycles lasting 24 to 31 days. None were pregnant, breastfeeding or on hormonal therapy during the two months prior to and during the study. No change in body weight was reported during the study period.

Specimen collection

The protocol required each subject to provide one morning spot and one overnight urine sample per menstrual cycle during the six-month period. Five participants missed one sample during the study period and one subject missed two samples. Three women collected their urine samples on the same "numerical" day of the follicular phase (days six to eight counting the first day of the menstrual bleeding as the first day of menstrual cycle), two women collected the samples on the same "numerical" day of the luteal phase (days 20 to 24), and two women collected urine in the time between the late luteal and the early follicular phases (days 27 to three) as a result of early menstrual periods. The overnight urine collection protocol called for discarding of the last void before going to bed and collection of all urine voided during the night including the first void of the morning. Overnight urine samples were kept at room temperature. Completeness of the overnight urine collection was determined by asking the participants about the quantity of urine lost during the collection. All participants reported accurate overnight urine collection. Morning spot urine samples were collected when participants delivered the overnight urine samples between 8:00 and 10:00 am to the Center for Preventive Medicine in the Department of Social and Preventive Medicine at State University of New York at Buffalo, NY. No preservatives were added to the urine. At the Center, samples were processed soon after the delivery of the overnight sample and the collection of the morning spot sample. The urine samples were filtered, and 1 mL aliquots were stored at -70°C. All samples collected over the six-month period were taken out of the freezer simultaneously and sent in the same parcel on dry ice to the laboratory for the hormonal determinations.

Laboratory methods

All samples from each subject were assayed simultaneously in the same run by the same laboratory technician, and in a blind fashion. Estrone-3-glucuronide was measured using 100 µL of urine mixed with 1.9 mL of assay buffer (0.05 M phosphate/EDTA buffer with 0.1% sodium azide and 0.1% bovine serum albumin) to give a dilution of 1:20 (v/v). Aliquots were assayed in duplicate. Standard solutions were prepared containing 2,000, 1,000, 500, 250, 125, 62.5, 32.25 and 15.125 pg of estrone-3-glucuronide/0.1 mL of assay buffer.

Standards and samples were mixed with tritiated estrone (100 µL containing approximately 22,000 dpm/31 pg) and with antiserum (suitably diluted in 100 µL of buffer). The total volume of the incubation mixture was brought to 500 µL with addition of buffer, thoroughly mixed, and incubated at 4°C (18h and 4h for the first and the second experiment respectively). The antibody-bound and free steroids were separated with 0.5 mL of dextran-coated charcoal (2.5

g of charcoal and 0.25 g of dextran T70 per liter) maintained in suspension with a magnetic stirrer. After equilibration for 15 minutes at 4°C, the mixture was centrifuged for 15 minutes at 10°C at 2,000 g. The supernatant was decanted into a polypropylene vial containing 4 mL of scintillation fluid (Pico-Fluor 40 from Packard). The solutions were mixed and the absolute amount of radioactivity was determined by counting each vial for 10 minutes. The unknown concentrations of samples were derived from the standard curve by an automatic program based on the 4PL algorithm, and the values multiplied by 0.2 to give the results in ng/mL urine (to convert the ng/mL in SI units - nmol/L - multiplied by 2.24).

Pregnandiol-3-glucuronide was measured using 100 µL of urine mixed with 1.9 mL of the same assay buffer to give a dilution of 1:400 (v/v). The dilutions used for the estrone-3-glucuronide determinations were employed.

Aliquots (50 µL and 100 µL for luteal and follicular phase respectively) were assayed in duplicate. Standard solutions were prepared containing 2,000, 1,000, 500, 250, 125, 62.5, 32.25 and 15.125 pg of 5β-pregnanediol-3α-glucuronide/0.1 mL of assay buffer. Standards and samples were mixed with tritiated 20α hydroxyprogesterone (100 µL containing approx. 11,000 dpm/53 pg) and with antiserum (suitably diluted in 100 µL of buffer). The total volume of the incubation mixture was brought to 500 µL with additional buffer, thoroughly mixed, and incubated at 4°C (18h and 4h for the first and the second experiment respectively). The antibody-bound and free steroids were separated with 0.5 mL of dextran-coated charcoal (2.5 g of charcoal and 0.25 g of dextran T70 per liter) maintained in suspension by a magnetic stirrer. After equilibration for 15 minutes at 4°C, the mixture was centrifuged (15 minutes at 10°C at 2000 g) and the supernatant decanted into a polypropylene vial containing 4 mL of scintillation fluid (Pico-Fluor 40 from Packard). The solutions were mixed and the absolute amount of radioactivity determined by counting each vial for 10 minutes. The unknown concentrations were derived from the standard curve by an automatic program based on the 4PL algorithm and the values multiplied by 8 and 4 for luteal and follicular samples respectively to give the results in ng/mL urine (to convert the ng/mL in SI units - nmol/L - multiplied by 2.24).

The intra-assay analytical variability, expressed as a coefficient of variation percentage (CV %), was measured by using commercial lyophilized control serum at different concentration levels (Lyphochek; Bio-Rad, Milan, Italy). Quality control samples were added to unknown samples at the beginning, in the middle and at the end of each run to check for drifts in the assaying procedures. No drift of the control values was observed, indicating no obvious systematic error during the measurements. The results are presented in Table 1.

Statistical methods

Means and standard deviations for urine hormone determinations were computed for each subject. Estimates of variance components (within and between variance) and the intraclass correlation coefficients (ICC) were calculated according to Fleiss (22). To indicate the lower limit of uncertainty of the degree of reliability, the lower limit of the 95% confidence interval of the intraclass correlation coefficients was calculated in addition to the point estimate (22). The minimum number of replicate measurements needed to correctly estimate the hormone level after setting a desired reliability level of 0.90 was computed using

TABLE 1
Analytical variability of hormone assays

Hormone level (mean level of control sera)	Intra-assay variability	
	number of determinations	CV (%)*
Estrone-3-glucuronide (ng/mL)		
17.59	4	7.7
34.61	4	5.9
Pregnandiol-3-glucuronide		
634.45	4	18.3
2185.29	4	9.9
9168.58	4	11.9
19521.29	4	10.1

* coefficient of variation = (SD/mean) x 100

the Spearman-Brown formula (22). The degree of linear association between urinary hormone metabolites measured in the morning spot and in the overnight urine samples was analyzed with the Pearson correlation coefficient, using the complete data across all subjects and all replicates. The software package used for descriptive data and the correlations was the Statistical Package for Social Sciences for Windows (version 7) (23).

Results

Means and standard deviations for urinary estrone-3-glucuronide and pregnandiol-3-glucuronide measured in morning spot and overnight collections across the replicates are shown for each participant in Tables 2 and 3 respectively. Hormone metabolites were generally more concentrated in the morning spot urine (24). Specimens for subjects 1 and 5 were collected during the luteal phase and showed a high concentration of the steroid metabolites, with pregnandiol presenting the highest levels as expected. Specimens for subjects 2 and 3 were collected between the late luteal and the early follicular phase, a period during which progesterone changes from the highest to the lowest serum level in the menstrual cycle. Concentration of pregnandiol in urine follows the changes in concentration of its serum precursor. Consistent with this pattern, subjects 2 and 3 showed intermediate concentrations of pregnandiol glucuronide.

Table 4 shows the estimates of variance components, the reliability coefficients, their lower 95% confidence limit and the number of replicates necessary for reliable estimation of estrone-3-glucuronide and pregnandiol-3-glucuronide measurements in morning spot and overnight urine samples. For estrone and pregnandiol, and for both morning spot and overnight urine collections, between-subject variance was larger than within-subject variance, indicating good reliabilities of their measurements.

A higher coefficient was observed for estrone and pregnandiol in the morning spot samples. Estrone-3-glucuronide displayed a large difference in the coefficients between morning spot and overnight urine collection with ICCs of 0.78 and 0.47 respectively. The Spearman-Brown formula indicated that three replicates would be required for both urinary metabolites using morning spot samples, and ten and five replicates for estrone and pregnandiol determinations in overnight samples.

Lastly, estrone-3-glucuronide and pregnandiol-3-glucuronide concentrations in the morning spot urine showed good correlation with overnight concentrations (Pearson's r =0.7 and 0.8 respectively).

Discussion

In the last twenty years, urine has been regarded as a significant specimen for collection in population-based studies of breast cancer and other chronic diseases.

Large epidemiological studies that collect and store specimens in biological banks (25) have renewed this interest in urine samples, since they have the advantage of being non-invasive and easy to collect, while new laboratory methods have been adapted to urine and formatted into inexpensive assays particularly suitable for epidemiological and clinical studies on large number of subjects (26). However, information concerning the intra-individual variability of urinary metabolites is still limited, particularly for hormone metabolites in premenopausal women. To satisfactorily test

Subject	Number of samples	Morning spot urine sample: mean (SD) level of estrone (ng/mL)	Overnight urine sample: mean (SD) level of estrone (ng/mL)
1	5	76.22 (24.7)	58.62 (32.6)
2	4	31.55 (13.3)	11.19 (5.2)
3	5	25.96 (6.7)	10.88 (4.1)
4	6	16.29 (11.1)	23.37 (10.4)
5	5	44.98 (7.8)	24.16 (6.1)
6	5	13.21 (5.9)	23.42 (17.0)
7	6	11.48 (6.8)	21.00 (7.9)

TABLE 2
Mean (± SD) of estrone-3-glucuronide from morning spot and overnight urine collection for each of the seven study participants

Subject	Number of samples	Morning spot urine sample: mean (SD) level of pregnandiol (ng/mL)	Overnight urine sample: mean (SD) level of pregnandiol (ng/mL)
1	5	8901.6 (2241.2)	6681.8 (3192.8)
2	4	4635.5 (3102.2)	1981.3 (2179.8)
3	5	2847.4 (2308.3)	1309.2 (919.7)
4	6	809.3 (543.2)	903.8 (436.2)
5	5	4099.6 (893.7)	2323.4 (680.9)
6	5	1042.5 (369.8)	1627.6 (1321.0)
7	6	772.1 (733.1)	1307.3 (535.1)

TABLE 3
Mean (± SD) of pregnandiol-3-glucuronide from morning spot and overnight urine collection for each of the seven study participants

for an association between physiologic variables and risk of diseases, individual subjects must first be adequately characterized. This task is particularly difficult for hormone metabolites in young women due to circadian and monthly fluctuation of hormone precursors in serum, as well as other factors contributing to hormone metabolism and excretion, such as cigarette smoking or medication intake.

The urinary steroid metabolite measurements included in this study showed moderate to good reliability with ICC ranging from 0.47 (estrone - overnight) to 0.78 (estrone - morning spot). As expected, measurements during the luteal phase had the highest level of urinary pregnandiol.

Measurements of the urinary metabolites of progesterone and especially estrogens were found to be more reliable in the morning spot urine, perhaps because concentrations tended to be higher in the morning spot, as found by

TABLE 4
Estimate of variance components, intraclass correlation coefficient (ICC) and lower limit of the 95% confidence interval (95% CI) for estrone-3-glucuronide and pregnandiol-3-glucuronide in morning spot and overnight urine samples

Hormones	Estimate of variance components	ICC	95% CI (lower-bound)	Minimum number of replicates
Estrone-3-glucuronide				
Morning spot samples				
Between subjects	17896.4			
Within subjects	4415.0	0.783	0.579	3
Overnight urine samples				
Between subjects	7539.1			
Within subjects	6592.0	0.468	0.200	10
Pregnandiol-3-glucuronide				
Morning spot samples				
Between subjects	268182570.1			
Within subjects	79190011.0	0.752	0.531	3
Overnight urine samples				
Between subjects	121087501.9			
Within subjects	56615388.5	0.645	0.388	5

others (24), with the result that the intra-assay variability was lower. Higher morning levels may be related to the circadian rhythm of hormones. Panico *et al* (27), for example, reported more elevated levels of estrogen and progesterones in the morning. The good correlation between the morning spot and the overnight estrone and pregnandiol levels suggests that the morning spot urine could be an alternative to the more complex overnight urine collection in the epidemiological evaluation of urinary hormonal profiles.

The small sample size and the missed collections at one time point for five of the seven women are the major limitations of this study. However, it appears to be the first investigation in which the reliability of urinary hormone metabolites over a short period of time was evaluated while checking for several sources of biological and methodological variability using strict inclusion criteria and highly standardized urine collection procedures, including urine collection between 8:00 and 10:00 *am*, restriction of urine collection to the same menstrual phase, exclusion of participants taking hormone therapy, and assaying all samples from the same person at the same time to eliminate the effect of the inter-assay variability.

This study was conducted to assess the consistency of two hormone metabolite determinations from urine samples in a group of seven healthy premenopausal women over a six-month period. Variations due to circadian rhythm and inter-assay laboratory variability were deliberately minimised. Under these conditions, estrone-3-glucuronide and pregnandiol-3-glucuronide levels in urine morning spot showed good reliability. Replication of this study with a larger sample, coupled with evaluation of the consistency and predictability of determinations of urinary hormone metabolities and their precursors, would result in better characterization of individual risk profiles and a clearer understanding of the role of such metabolites in the etiology of breast cancer.

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Effects of Transportation and Delay in Processing on the Stability of Nutritional and Metabolic Biomarkers

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The abbreviations are: Alk Phos, Alkaline Phosphatase; CI, Confidence Interval; CV, coefficient of variation; GSH, glutathione; GSHpx, glutathione peroxidase; GGTP, gamma glutamyltransferase; LDL/VLDL, low density lipoprotein cholesterol/very low density lipoprotein cholesterol; PRBC, packed red blood cells; SGOT, serum aspartate amino transferase; SGPT, serum alanine aminotransferase; TBARS, thiobarbituric acid-reactive substances; TEAC, trolox equivalent antioxidant capacity

ABSTRACT

The effects of transportation and delay in processing of blood samples on the concentration of biomarkers is significant to epidemiological studies that collect specimens from participants at locations other than a designated center or laboratory. These sources of variability in measurement were studied by collecting two sets of blood samples from 51 men between the ages of 26 and 50. The first set was sent immediately to the laboratory for processing. The second set was transported by car for one hour and then returned to the laboratory for processing. Both sets were stored together at –80° C until the end of the study. Several blood constituents were evaluated. Vitamins, liver enzymes, and electrolytes showed no changes in concentration after transport by car for one hour. There were decreases in the concentrations of red and white blood cells, HDL cholesterol, glucose, and creatinine after transportation. The transported total cholesterol, total testosterone, free testosterone, alkaline phosphatase, total bilirubin, and TBARS increased in concentration. Although transportation and delay in processing of blood samples do not appear to greatly impact relative risk estimates, epidemiologists should be aware of these potential sources of variability in measurement and consider the consequences in their particular study.

INTRODUCTION

The effects of transportation and delay in processing of blood on the concentration of biomarkers is significant to epidemiological studies that collect specimens from participants at locations other than a designated center or laboratory. Some participants, particularly older and sicker individuals, may be more likely to have their blood collected at home than healthier participants. If transportation or delay in processing of the samples were to affect the concentration of the biomarkers of interest, there could be a distortion in the comparison of participants with particular characteristics. Concern for such an effect is what led to this study. In a series of case-control studies conducted in Erie and Niagara Counties on alcohol intake and chronic disease, researchers recognized that cancer cases were more likely to have their blood collected at home and transported to the study center, while healthy controls were more likely to have their blood collected at the study center, in the same building the blood was processed.

A study was designed to investigate the short-term (one hour) effect of transportation by car and delay in processing on the levels of several blood constituents in serum and plasma while controlling for other sources of variability. Since the aim of the study was to validate specific collection procedures for specimens (home versus on-site), the effects of transportation and delay in processing are considered together as a potential factor affecting serum levels of nutritional and metabolic biomarkers.

MATERIALS AND METHODS

Specimen collection and handling

Fifty-one healthy men between the ages of 26 and 50 were recruited for this study. Two sets of blood samples were collected from each participant by a trained phlebotomist. All samples were collected between 6 am and 10 am after a twelve hour fast. Each set consisted of 45 ml of blood collected in five tubes, drawn in the following order: a 10 ml red top with no additive, a 10 ml green top with sodium heparin, a 10 ml purple top with EDTA, a 5 ml purple top with EDTA, and an additional 10 ml red top with no additive. A second identical set was drawn immediately after the first. For each set, the temperature of the room and the time the first tube of blood was drawn was recorded. As each tube was drawn it was placed in a foil jacket to protect it from light. Each tube was handled according to specific

processing protocols.

The first set was handled as described below. The green and purple top tubes from the first set were wrapped in paper towels and placed in a cooler with an ice pack. The two red top tubes were wrapped in paper towels and kept at room temperature to allow for clotting. This set was then immediately sent to the processing laboratory. The time difference between the blood draw and the time of processing was recorded. The small purple top tube was placed in a cooler for determination of blood cell counts at a local hospital laboratory. The red top tubes were allowed to clot at room temperature for exactly 30 minutes then centrifuged at 4° C at 3,000 rpm for 10 minutes. After this step, the aliquots of serum and plasma were kept at room temperature waiting for the second set to arrive.

All tubes for the second set were wrapped in paper towels and placed in a large biohazard bag. This bag was placed along with a gel freeze pack in a styrofoam cooler with a tight fitting lid. The samples were then transported for one hour by car. After transportation, the samples were sent to the processing laboratory and the time difference between the blood drawing and processing was recorded. The small purple top tube was placed in the cooler along with the tube from the first set. The remaining tubes were processed identically to the first set. Both sets were then stored at –80° C until the analytical determinations.

Laboratory methods

For each participant, samples from the two sets were assayed simultaneously in the same run, in a blind fashion, and by the same laboratory technician. While 51 pairs of samples were collected, for some biomarkers determinations could not be performed because a sufficient amount of sample was not available. The number of pairs analyzed for each biomarker are indicated. All parameters had an intra-assay coefficient of variation (CV) less than 9 percent, while most were less than five percent.

Retinol, α-tocopherol and carotenoids were measured in serum simultaneously by high performance liquid chromatography with photodiode array detection (1). Total ascorbic acid was determined by the dinitrophenylhydrazine method (2) after it was stabilized by adding 0.5 ml of heparin

plasma to 2.0 ml of 6% meta-phosphoric acid and centrifuged at 3,000 rpm for 10 minutes. Erythrocyte glutathione (GSH) was determined by fluorometric analysis using o-phthalaldehyde (3) after stabilization in meta-phosphoric acid stabilization buffer containing 1.67 g/dl MPA, 200 mg/dl EDTA and 30% sodium chloride.

Complete blood count (CBC) and serum chemistry profile were performed at a local hospital laboratoy using a Coulter Counter (Coulter Corp., Miami, FL) and a Paramax automated chemistry analyzer (Dade International, Inc., Aguada P.R. USA) respectively. Red and white blood cell counts were determined for 49 pairs of samples; platelets were determined for 46 pairs. Serum chemistry profiles were determined for 50 pairs of samples.

Total cholesterol was determined as part of the serum chemistry profile of a hospital laboratory (total cholesterol-H) and on plasma by our university laboratory (total cholesterol-U). At the university laboratory, cholesterol in lipoprotein fractions and sub-fractions were determined according to the method of Gidez et al (4) on a Cobas Mira automated chemistry analyzer (Roche Diagnostics Corp. Indianapolis, IN) using reagent kits from Sigma Chemical Co., St. Louis, MO. Lipid determinations were performed for 50 pairs of samples.

Free testosterone was assayed by coated-tube RIA with a "coat-a-counts" kit from Diagnostic Products Corporation, Medical System (Genova, Italy). Total testosterone was assayed with a non-extraction, double-antibody RIA kit from Sorin Biomedica (Saluggia, Italy). Insulin was assayed with a non-extraction RIA kit from Ares Serono Diagnostics using a polyethylene glycol solution for the free/bound separation. Hormone determinations were performed for 50 pairs of samples.

Plasma thiobarbituric acid reactive substances (p-TBARS) were measured according to Armstrong and Browne (5) and expressed in nmol/mL of malondialdehyde equivalents. Low density lipoprotein TBARS (LDL/VLDL-TBARS) were determined on manganese chloride/heparin precipitates from lipoprotein isolation (6). Trolox equivalent antioxidant capacity (TEAC) was measured according to the procedure described by Miller et al (7). TEAC was determined for 44 pairs of samples. Plasma glutathione peroxidase (GSHpx) was determined by automated enzyme kinetic assay on the Cobas Mira

analyzer (8). GSHpx was determined for 46 pairs of samples.

Statistical methods

Descriptive statistics included the mean and standard deviation for each biomarker. Histograms were used to compare the distributions of the non-transported and transported samples. Statistical testing was performed using a paired t-test for each biomarker to compare the non-transported and transported samples. The potential effects of time to processing, baseline serum levels of endogenous preservatives, namely vitamin C, vitamin E, and total bilirubin, and baseline serum level of glucose as a potential degrading agent were tested using linear regression. All statistically significant variables were tested for normality using the Kolmolgorov Smirnoff test. Using the enter method, linear regression was then performed on biomarkers with t-tests having a p-value < 0.05. The paired difference for each biomarker was used as the dependent variable. The time to processing, and the baseline concentrations of vitamin C, vitamin E, total bilirubin, and glucose were used as independent variables. In order to evaluate a potential differential effect of transportation (or delay in processing) by serum levels of the analytes, we performed cross-tabulations of the transported and non-transported samples that had been categorized into quintiles. We then tested the consistency of individual characterization across quintiles of transported versus non-transported samples using X^2 test.

To illustrate what effect transportation and delay in processing could have on relative risk estimates, the intraclass correlation coefficient (ICC) was calculated by taking the difference of the between mean square and the within mean square and dividing this difference by the sum of the between mean square and the within mean square (9). An observed relative risk was then calculated by multiplying the natural log of a specific true relative risk (i.e. 1.5 and 2.0) by the intraclass correlation coefficient and taking the antilogorithm of this product (10).

RESULTS

Because it is possible to obtain statistical significance by chance given the number of tests peformed in this study on a variety of biomarkers, we present the biomarkers in specific groups in an effort to find biological reasons for results observed.

For each biomarker, the mean for the non-transported sample, the direction of change, the mean difference of the measures and the corresponding 95% confidence intervals are presented in tables 1 through 4.

There were negligible changes in concentrations for both fat soluble vitamins and vitamin C and all 95% confidence intervals included zero (data not shown).

The red blood cells, white blood cells, and platelets decreased after transport by car for one hour (Table 1). Table 2 presents values for lipids and hormones. Both laboratory determinations of total cholesterol showed an increase in concentration in the transported sample. HDL cholesterol decreased in the transported sample. Both total testosterone and free testosterone were higher in the transported sample. Insulin was unchanged. The 95% confidence intervals of the paired differences for total cholesterol-H, HDL cholesterol, and total testosterone did not include zero.

The oxidative stress indicators are presented in Table 3. The oxidative stress parameters that are indicators of damage (TBARS and LDL/VLDL-TBARS) showed an overall increase in concentration after transportation. Conversely, indicators of protection (GSH, GSH-px, and TEAC) showed an overall decrease.

In Table 4 are presented several biomarkers that were part of the serum chemistry profile. After transportation, measures of glucose and creatinine concentration were lower, while alkaline phosphatase and total bilirubin levels increased. The 95% confidence intervals for all four of these components did not include the null. Liver enzymes (GGTP, SGOT, and SGPT) and electrolytes showed no meaningful changes.

The time to processing and baseline concentrations of vitamin C, vitamin E, total bilirubin, and glucose did not explain a significant amount of variation observed in the biomarkers (data not shown). In order to evaluate whether there was misclassification by serum level of each analyte, we performed cross-tabulations of the transported and non-transported samples that had been categorized into quintiles. Overall, we observed that the misclassification occurred within one quintile and the observed differences across categories of transported versus non-transported samples were not statistically significant.

The intraclass correlation coefficients for blood cells, lipids and hormones were all greater than or equal to 0.90, with a lower limit of 0.86. Most were above 0.95. The intraclass correlation coefficient for free testosterone was 0.90 (CI 0.86-0.94). Applying this ICC to the formula outlined previously (10), one would actually observe relative risks of 1.44 and 1.86 for true relative risks of 1.5 and 2.0, respectively.

DISCUSSION

This study was conducted to investigate the effect of transportation by car and delay in processing on the measured concentration of biomarkers in blood while controlling for several sources of variability. Vitamin, liver enzyme, and electrolyte measurements did not show any significant changes after transportation. However, our findings suggest that a transportation effect and the associated delay in processing of samples should be considered as potential sources of variability in the measurement of other biomarkers. Counts of red blood cells and white blood cells, and the measured concentrations of glucose and creatinine decreased after transportation. Measurements of total cholesterol, total testosterone, free testosterone, alkaline phosphatase, total bilirubin, and TBARS increased.

The carotenoids and vitamins A, C, and E remained stable after transport by car for one hour. There have been previous reports on the stability of vitamins such as carotenoids, retinol, and tocopherol in both serum and plasma when held at various temperatures for up to five days (11, 12). Hankinson et al. (13) investigated the effect of transport by mail on samples of blood that were immediately centrifuged and then mailed versus samples that were shipped as whole blood. In that study, they also found retinol to be stable in samples when mailed as cooled whole blood; however α-tocopherol and β-carotene measures were significantly lower in cooled whole blood than in centrifuged blood.

Transportation with associated cell lysis and/or delay in processing of samples with associated continued metabolic activity of the survived cells may directly explain many of the differences observed. Blood cell counts decreased after transportation. In general, red blood cells tend to lyse and clot both during venipuncture and with a delay in processing. White blood cells tend to adhere to blood tube surfaces and to each other. However, it is more likely that the decrease in blood cells observed was due to a transportation effect and not related to venipuncture or delay in processing for this determination. Other

than the transportation, the non-transported and transported tubes were handled identically. The time to processing was the same for both sets.

Cell lysis may explain many of the other differences observed. Lysis of cells could increase extracellular cholesterol and explain the overall increase in total cholesterol. Upon lysis, cells release a host of enzymes, including lipoprotein lipase and alkaline phosphatase, that are relatively concentrated in the red and white blood cells. The decrease in HDL cholesterol in the transported sample may in turn be explained by an increase in extracellular lipoprotein lipase activity. Lipoprotein lipase affects the structure of the lipoproteins resulting in the formation of micelles. The formation of micelles may interfere with the lipid soluble steroid determinations and be responsible for the observed increase in total and free testosterone levels. Similar increases in testosterone concentration have been reported by others. Hankinson et al. (13) found that testosterone levels continued to increase as the time to processing increased for up to 48 hours after blood draw. Berrino et al. (14) reported that free testosterone was significantly higher in samples that had been transported than in samples that had been collected and processed in the same building.

Hemoglobin is also released during cell lysis, and is metabolized through a series of intermediates to bilirubin which accumulates in the sample. But the continued activity of the survived cells may explain other results. The red blood cells that do not lyse continue to metabolize glucose for energy. The red blood cells consume oxygen and produce carbon dioxide and lactate decreasing the pH. This decrease in pH of the sample can result in the deamination of nitrogenous compounds such as creatinine, the dissociation of a molecule from its binding or transport protein, or alterations in protein structure.

The changes observed for oxidative stress indices are consistent with increased oxidation by cell lysis. Overall, the oxidative stress indicators that are markers of damage (TBARS, LDL/VLDL-TBARS) showed an increase, whereas the markers of oxidative protection (GSH, GSHpx, TEAC) showed a decrease; this overall decrease in the indicators of oxidative stress protectors might be a related compensatory effect in response to cell damage. Hemoglobin released as a result of red blood cell lysis is a source of ferrous ions which generate hydroxyl radicals in the presence of hydrogen peroxide.

Hydroxyl radical is a powerful oxidant which can peroxidize lipids and may explain the observed increase in plasma TBARS. The lipids within the LDL particle of LDL/VLDL-TBARS may not show this increase because of their protected environment within the lipoprotein particle, rich in fat-soluble antioxidant vitamins E and carotenoids. It is important to note these biomarkers of oxidative stress are affected by relevant analytic limitations (15). New and more direct measures of oxidative stress damage could help corroborate the suggested evidence of an increase in oxidative stress damage with transportation and delay in processing.

No changes in the concentration of electrolytes were found after transport by car for one hour. Others also found sodium, potassium, and calcium to be stable by mail for up to four days (16).

We estimated what effect transportation and delay in processing could have on true relative risk estimates in epidemiological studies. In this study, we found changes in concentration mainly among the blood cells, lipids and hormones. Using the intraclass correlation coefficient for free testosterone we demonstrated a mild attenuation of relative risks of 1.44 and 1.86 for true relative risks of 1.5 and 2.0, respectively. For biomarkers with a higher intraclass correlation coefficient (i.e. 0.95), there would be little effect on relative risk estimates. This is assuming random misclassification.

A limitation of our study is that it is not possible to separate an effect due to transportation from an effect due to the delay in processing of the samples. Centrifuging samples prior to transport would differentiate these two potential effects. Others have reported serum cholesterol, creatinine and alkaline phosphatase to be stable at various temperatures and for up to four days when the sample was centrifuged immediately (16, 17). However, centrifugation is not feasible in studies that collect samples from sites other than the laboratory. Therefore, separating an effect due to presence of cells for a longer time period from a transport effect is not in itself important to those epidemiological studies. Rather, it is more important to identify and quantitate this source of error.

Another limitation is a potential for a systematic difference in the way the samples were collected. The two sets of samples were collected consecutively to reduce the intra-individual variability, and the second set was always transported. It may be that the second set had a higher concentration of

nonfilterable blood constituents due to hemoconcentration resulting from prolonged application of a tourniquet. However, the observed decrease in blood cells is not consistent with this explanation.

Also, all transported tubes of blood were placed in a cooler containing a gel freeze pack. However, the red top tubes from the first set were kept at room temperature, which is standard protocol. After a home draw visit the red top tubes are kept at room temperature rather than being placed in a cooler with the other blood tubes. Since our study was conducted during the summer months and the average daily temperature varied, it was decided to place the red top tubes in the cooler so that the temperature of the car would not be an additional source of variability. It is possible to argue that the biomarkers are more stable at room temperature than in a cooler, or vice versa.

There are several strengths to this study. The study was designed to limit many sources of variability associated with an accurate measure of biomarkers. For each participant, the second set of blood was drawn immediately after the first to reduce intra-individual variability. Each person was also used as his own control. Both sets of blood drawn for each participant were frozen at the same time and kept at –80° C until the end of the study. Each participant's samples were then assayed in the same run, in a blind fashion and by the same laboratory technician to reduce laboratory variability.

The importance of transitional studies to investigate the reliability, validity, and feasibility of the biomarkers in research has been discussed by several authors (18 - 24). Despite the need for these studies, there are few reports in the published literature as to the effect of transportation on samples of blood (13, 16, 17, 25, 26). In fact, we found no other reports in the literature of actual transport of samples by car for biomarkers we include in our study.

The results from this study suggest that transportation and delay in processing are potential sources of variability in the measurement of some biomarkers, such as hormones. It appears the actual transportation is responsible for some differences, particularly for blood cells. For other biomarkers it is unclear whether the transportation (associated with cell lysis) and/or delay in processing of samples (associated with continued metabolic activity of the cells) are responsible for the differences observed. In general, this source of variability does not appear to greatly impact relative risk estimates. Nevertheless,

because epidemiologists frequently deal with small increases in relative risks, they should be aware of this potential source of variability in measurement and consider the consequences of this variability for their particular study.

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Table 1. Change in concentration of blood cells after transportation by car for one hour in a study of 51 men conducted at the University at Buffalo, New York, June-July 1997.

Variable (unit)	No. of samples	Non-Transported Mean (SD)[a]	Mean of Paired Difference[b]	95% CI of Paired Difference[c]
Red Blood Cell Count (10 x 6)	49	4.97 (0.39)	0.027	(-0.00063, 0.054)
White Blood Cell Count (10 x 3)	49	5.99 (1.80)	0.14	(0.00018, 0.28)
Platelets (10 x 3)	46	200.30 (37.14)	1.13	(-1.73, 3.99)

[a] The mean concentration and standard deviation of the non-transported sample.

[b] Paired difference is calculated from paired t-test using mean of non-transported sample minus mean of transported sample.

[c] CI, confidence interval.

Table 2. Change in concentration of lipids and hormones after transportation by car for one hour in a study of 51 men conducted at the University at Buffalo, New York, June-July 1997.

Variable (unit)	No. of samples	Non-Transported Mean (SD)[a]	Mean of Paired Difference[b]	95% CI[c] of Paired Difference
Total Cholesterol-H (mg/dl)	50	195.2 (43.2)	-2.8	(-4.8, -0.9)
Total Cholesterol-U (mg/dl)	50	196.5 (45.0)	-0.5	(-3.1, 2.1)
HDL[c] Cholesterol (mg/dl)	50	68.0 (10.6)	1.3	(0.2, 2.5)
Triglycerides (mg/dl)	50	109.5 (52.4)	-1.3	(-3.5, 0.8)
Total Testosterone (pg/ml)	50	6.3 (2.1)	-0.2	(-0.4, -0.03)
Free Testosterone (ng/ml)	50	14.3 (5.0)	-0.4	(-1.0, 0.3)
Insulin (μg/ml)	50	9.3 (8.7)	-0.08	(-0.7, 0.5)

[a] The mean concentration and standard deviation of the non-transported sample.

[b] Paired difference is calculated from paired t-test using mean of non-transported sample minus mean of transported sample.

[c] CI, confidence interval; HDL, high density lipoprotein.

Table 3. Change in concentration of oxidative stress indicators after transportation by car for one hour in a study of 51 men conducted at the University at Buffalo, New York, June-July 1997.

Variable (unit)	No. of samples	Non- Transported Mean (SD)[a]	Mean of Paired Difference[b]	95% CI[c] of Paired Difference
TBARS[c] (nmol/ml)	51	1.16 (0.19)	-0.033	(-0.066, 0.0063)
LDL/VLDL[c] -TBARS (nmol/ml)	51	0.37 (0.051)	-0.0017	(-0.0098, 0.0065)
Glutathione (mg/dl PRBC[c])	51	42.18 (7.27)	2.52	(-0.33, 5.37)
GSHpx[c] (IU)	46	611.15 (55.94)	4.59	(-7.34, 16.51)
TEAC[c] (%)	44	75.26 (2.46)	1.19	(-0.10, 2.48)

[a] The mean concentration and standard deviation of the non-transported sample.

[b] Paired difference is calculated from paired t-test using mean of non-transported sample minus mean of transported sample.

[c] CI, confidence interval, TBARS, thiobarbituric acid-reactive substances; LDL/VLDL, low density lipoprotein cholesterol/very low density lipoprotein cholesterol; PRBC, packed red blood cells; GSHpx, glutathione peroxidase; TEAC, trolox equivalent antioxidant capacity.

Table 4. Change in concentration of serum constituents after transportation by car for one hour in a study of 51 men conducted at the University at Buffalo, New York, June-July 1997.

Variable (unit)	No. of samples	Non-Transported Mean (SD)[a]	Mean of Paired Difference[b]	95% CI[c] of Paired Difference
Glucose (mg/dl)	50	93.8 (9.7)	2.9	(2.0, 3.7)
Creatinine (mg/dl)	50	1.0 (0.1)	0.03	(0.02, 0.05)
Alkaline Phosphatase (u/l)	50	63.0 (15.0)	-0.7	(-1.2, -0.2)
Total Bilirubin (mg/dl)	50	0.8 (0.4)	-0.03	(-0.05, -0.02)
GGTP[c] (u/l)	50	30.7 (20.2)	19.2	(-19.7, 58.1)
SGOT[c] (u/l)	50	27.6 (10.1)	0.4	(-0.4, 1.1)
SGPT[c] (u/l)	50	24.9 (25.8)	0.08	(-0.8, 0.9)
Calcium (mg/dl)	50	9.6 (0.3)	0.04	(-0.03, 0.1)
Sodium (mmol/l)	50	142.3 (1.7)	0.2	(-0.4, 0.7)
Potassium (mmol/l)	50	4.2 (0.3)	-0.01	(-0.07, 0.04)

[a] The mean concentration and standard deviation of the non-transported sample.

[b] Paired difference is calculated from paired t-test using mean of non-transported sample minus mean of transported sample.

[c] CI, confidence interval, GGTP, gamma glutamyltransferase; SGOT, serum aspartate amino transferase; SGPT, serum alanine aminotransferase.

Table A. Percent change in concentration of lipids and hormones after transportation by car for one hour in a study of 51 men conducted at the University at Buffalo, New York, June-July 1997.

Variable (unit)	No. of samples	Non-Transported Mean (SD)[a]	Percent Change[b]	95% CI[c] of Paired Difference
Total Cholesterol-H (mg/dl)	50	195.2 (43.2)	-1.4	(-2.5, -0.5)
Total Cholesterol-U (mg/dl)	50	196.5 (45.0)	-0.2	(-1.6, 1.1)
HDL[c] Cholesterol (mg/dl)	50	68.0 (10.6)	1.9	(0.3, 3.7)
Triglycerides (mg/dl)	50	109.5 (52.4)	-1.2	(-3.2, 0.7)
Total Testosterone (pg/ml)	50	6.3 (2.1)	-3.0	(-6.3, -0.5)
Free Testosterone (ng/ml)	50	14.3 (5.0)	-3.0	(-7.0, 2.1)
Insulin (μg/ml)	50	9.3 (8.7)	-0.9	(-7.5, 5.3)

[a] The mean concentration and standard deviation of the non-transported sample.

[b] The percentage change is calculated using the difference between the mean of non-transported sample minus the mean of the transported sample, and dividing this number by the mean of the non-transported sample.

[c] CI, confidence interval; HDL, high density lipoprotein.

Short-term storage of blood samples and DNA Isolation in Serum Separator Tubes for Application in Epidemiological Studies and Clinical Research[1]

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Abstract

The use of DNA for genetic analyses in population based studies is of increasing interest. These studies focus on large numbers of subjects and, therefore, require careful logistic planning of blood sample processing and storage as well as reliable methods. The overall goal is to obtain and isolate a large number of high quality DNA samples minimizing time, effort and costs. There is, however, limited information on a number of logistical factors that could influence the quantity and quality of DNA isolation in large scale studies. The present study analyzes systematically the potential impact of some of these factors: i.e., the lag time between blood drawing and DNA isolation and the condition of storage of blood samples on the quantity and quality of DNA isolated. A modified single tube DNA isolation technique was used. DNA was isolated from samples collected from 6 participants and processed: a) immediately after blood drawing, b) after blood cells were stored at 4°C for 7 days, c) after blood cells were stored at -80°C for 7 days, and d) after storage for 7 days at -80°C with addition of lysis/digestion buffer. All four conditions studied allowed isolation of highly pure DNA suitable for genetic analyses shown by Southern blot analysis and polymerase chain reaction. Storage at 4°C yielded, on the average, 20% less DNA than the samples processed immediately or after storage at -80°C. Our study indicates that storage for 7 days and at different temperatures does allow isolation of DNA of high quality. Storage of up to 7 days permits processing of large numbers of samples (50-70) in a single day, allowing for a reliable and cost efficient way of processing in various field settings.

Introduction

The use of DNA for genetic analyses in population based studies is of increasing interest in relation to etiology of cancer, cardiovascular and other diseases (1). Advancement in molecular biology techniques offers opportunities to study the correlation of phenotype and genotype in epidemiological studies. To develop rapid and safe procedures for DNA isolation is of particular interest for the use of DNA in biological specimen banks outlined by the National Heart, Lung, and Blood Institute working group in their recent guidelines on blood drawing, processing, and storage (1).

Most DNA isolation protocols rely on, and are complicated by, preisolation of white blood cells or buffy coat, a time consuming phase of DNA isolation (1, 2). Other rapid whole blood DNA isolation methods are not practical for use in large epidemiological studies (3). With more genetic and clinical epidemiological studies conducted it becomes more likely that genetic epidemiologists and clinical researchers will directly perform DNA isolation in their laboratories, since shipment of blood samples to other institutions diminishes control over a limited resource and enhances the potential for loss, misclassification, and misuse.

The processing of large number of specimens is improved by the development of new DNA isolation techniques that do not require preisolation of white blood cells or buffy coats. New DNA isolation techniques must be tested for utilization in large epidemiological studies. In large scale studies, batch processing of samples is often an important cost-saving procedure, but it requires storage of specimens for a period of time. However, limited information exists on the potential effect of lag time between

blood drawing and DNA isolation under various storage conditions. Madisen et al. (4), for example, reported decreased DNA yields within 3 days from cell pellets stored at 4°C or room temperature. These experiments were performed in samples from only one subject. Short-term storage of whole blood at - 70°C generally did not alter DNA yield but altered the size of isolated DNA (4).

This study was conducted to investigate systematically different conditions for short-term storage and their impact on DNA yield and purity. A recently reported method for DNA isolation was modified and tested for use in large scale epidemiological studies (5, 6). The protocol is based on an uncomplicated, inexpensive, and rapid procedure utilizing serum separator tubes (SSTs[4]) which can be made part of the laboratory routine. We also examined the effect of interruption of the isolation procedure at meaningful time points with completion after short-term storage to optimize timing of DNA isolation. These practical issues have to be considered to ensure economic and reliable procedures in epidemiological studies.

Materials and Method

Twelve fasting blood samples from each of 3 men and 3 women, aged 29 - 52, were drawn into citrate containing Vacutainers™ (Beckton Dickinson, Franklin Lakes, NJ) each holding 4.5 ml blood. An additional Vacutainer™ containing heparin was filled with blood from each subject for determination of white blood cell counts with a Coulter counter. Four different treatments were studied as shown in Figure 1: A) immediate isolation of DNA; B) storage at 4°C for 7 days; C) storage at -80°C for 7 days; D) addition of 1 ml lysis/digestion buffer to the cells, mixture, and storage at -80°C for 7 days. DNA was isolated after modification of a technique previously described (5, 6) based on single tube DNA isolation with a gel plug forming a barrier but using 10 ml plastic SSTs (Beckton Dickinson, Franklin Lakes, NJ).

DNA isolation

Whole blood, drawn by venipuncture, was transferred from citrate tubes to SSTs and plasma was removed after centrifugation of the samples at 2,000 rpm for 10 minutes at room temperature in a table top cell centrifuge. The gel plug forms a barrier closing the cellular fraction of blood beneath the plug. After removal of plasma, triplicate samples from each participant were assigned randomly to one of four experimental arms as depicted in Figure 1. In treatment A, samples were processed and DNA was isolated immediately as described below. In treatment B, a batch of samples from each subject was stored for 7 days at 4°C. For treatment C, samples were frozen at -80°C in SSTs for 7 days. Treatment D included addition of 1 ml digestion/lysis buffer (100 mM

NaCl, 100 mM Na_2EDTA, 50 mM Tris-Cl, pH 8.0, 3 % sodium dodecyl sulfate, 2 mg/ml proteinase K) to the cells and mixing before storage at -80°C for 7 days in SSTs.

One ml of digestion/lysis buffer was added immediately and mixed with the cells by carefully disturbing the plug interface with a clean Pasteur pipette (treatment A), after 7 day storage at 4°C (treatment B), after storage at -80°C and thawing at room temperature (treatment C), or before storage at -80°C (treatment D). The remaining steps of the protocol were the same for all treatments. Samples were incubated for 2 hr at 55°C in a waterbath. Then 2 ml of a 25:24:1 phenol:chloroform:isoamylalcohol mixture were added and samples were mixed by inversion. The aqueous phase containing the DNA was separated from the organic phase by centrifugation at 2,000 rpm for 15 minutes at 4°C with the gel plug forming a barrier between these phases. The aqueous phase remained on top of the gel plug. This step was repeated twice adding 1.5 ml of the phenol:chloroform:isoamylalcohol mixture to the same tube and centrifuging the samples for 10 min at 2,000 rpm. Subsequently, 2 ml of 24:1 chloroform:isoamylalcohol mixture were added to the tube before final centrifugation for 10 min at 2,000 rpm. After the final centrifugation the top aqueous layer was poured into a clean polypropylene tube and DNA was precipitated with an equal volume of isopropanol. The DNA was collected by spooling the precipitated DNA with a Pasteur pipette; it was air-dried and resuspended in 200 µl TE buffer (10 mM Tris-Cl, pH 8.0, 0.1 mM Na_2EDTA). Yield and purity of DNA were determined by measuring the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}), respectively, and using the A_{260}/A_{280} ratio as an indicator of purity.

We reduced the amount of organic solvents by approximately 15% in our protocol compared to that described by McIndoe et al. (6). Moreover, we reduced potential hazard to lab personnel and potential lossby use of plastic rather than glass SSTs minimizing the risk of breakage. Plastic tubes stored at -80°C exhibited occasionally slight discoloration but this change did not affect the integrity of the tube or DNA yield.

DNA electrophoresis and Southern blot analysis

Five μg of genomic DNA were digested with *BAM* HI restriction enzyme (BRL, Gaithersburg, MD), size-fractionated on 0.8 % agarose gels containing 1.0 μg/ml ethidium bromide, photographed, denatured, neutralized, and transferred to nitrocellulose membranes. DNA was immobilized by UV crosslinking. The probe used in this study was a 0.7 kb *Bam*H1 cDNA fragment encompassing exons 5 to 7 and 9 to 11 of the *MLL* gene on chromosome 11q23 (7). Southern blot analysis was performed with a ^{32}P-labeled probe that were generated by the random priming technique, using a Prime-It II kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol, and hybridized to nitrocellulose membranes as previously described (8). Final washing conditions were 0.1 % sodium dodecyl sulfate (SDS)/0.1 x SSC (1 x SSC = 0.15 mol/l NaCl and 0.015 mol/l sodium citrate) at 52°C. Autoradiography of blots was performed overnight at -70°C with an intensifying screen.

Polymerase Chain Reaction

A 469 bp fragment from exon 25 of the *MLL* locus on chromosome 11q23 was amplified using 20 mer-primers starting at nucleotide position 9214 (upstream primer) and 9682 (downstream primer) of the published sequence (9). PCR was performed using the GeneAmp PCR Reagent Kit and a Perkin Elmer Cetus Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT) according to the manufacturers recommendations. Final $MgCl_2$ and primer concentrations were 2.5 mM and 0.4 μM, respectively. Amplification conditions were 60 sec at 94°C, 60 sec at 57°C, and 90 sec at 72°C for 30

cycles. One-fifth of the reaction mixture was analyzed on 2.0 % agarose gel containing 1μg/ml ethidium bromide.

Statistical analysis

The mean and standard deviation for all considered variables were first computed for descriptive purposes for each subject and in each treatment group. Analysis of variance (ANOVA) with repeated measurements was applied to compare the difference in DNA yield and A_{260}/A_{280} per sample among the subjects in the treatment groups and among the 4 treatment groups. The software used was the Statistical Package for Social Sciences (10).

Results

DNA isolation

Mean DNA yields from triplicate samples per subject (n = 6) are presented in Table 1. The average amount of DNA isolated ranged from 27.0 to 71.1 μg/4.5 ml whole blood. Subject 3 showed significant lower DNA yields ($p < 0.05$) under all conditions; yield for subject 3 was only half of the average DNA amount isolated from subject 2. However, subject 3 also showed the lowest leukocyte (last column) and lymphocyte count (data not shown) among all subjects.

In Table 2 mean yield and purity of DNA are shown by treatment. The amount of DNA obtained from 4.5 ml whole blood averaged from 43.2 to 52.6 μg. Storage at 4°C for 7 days (treatment B) resulted in approximately 20 % less DNA than all other treatments, but this difference was not statistically significant ($p = 0.63$). A_{260}/A_{280} was 1.73 to 1.79 with no differences between treatments ($p = 0.56$).

DNA digest and electrophoresis

DNA is commonly analyzed by restriction digestion and Southern blot analysis. To properly analyze the pattern of DNA fragments produced by restriction digestion, it is essential that the DNA is completely digested with a minimum of non-specific degradation. Further, it is essential that the DNA be of high quality to obtain complete digestion. To demonstrate size and quality of DNA isolated after different storage conditions we performed agarose gel electrophoresis of undigested and restriction enzyme digested DNA (Figure 2). On the left hand side undigested DNA (2.5μg) from

treatment A, B, C, and D, respectively, is shown. Molecular weight markers (kb) are depicted in the center. Endonuclease-digested (*BAM* H1) samples from treatment A, B, C, and D, respectively, are shown on the right. Undigested DNA migrated as a single band above the 23 kb size standard, documenting an average DNA size of greater than 30 kb. Digested samples appeared with smear over a broad size range displaying the typical picture of completely digested genomic DNA. These data indicate that there was no difference in purity between the treatments and show the high quality of the DNA isolated with this technique.

Southern blot analysis and Polymerase Chain Reaction

We performed Southern blot analysis and PCR to explore if any of the treatments resulted in alteration of suitability of DNA for genetic analyses and if DNA isolated with this procedure is suitable for genomic analysis. The 0.7 kb *MLL* cDNA probe used detects a 8.3 kb germ line DNA fragment in Southern blot analysis of *BAM* H1 digested DNA of healthy normal individuals (7). Figure 3A shows a representative Southern blot analysis of genomic DNA obtained from the 4 different treatments (A, B, C, and D) and subsequently digested with *BAM* H1 and hybridized to the *MLL* cDNA probe. A single band of the expected 8.3 kb size can be observed in all four lanes while additional bands or smears which would indicate partial digestion with the restriction enzyme or general degradation are absent with all treatments.

Testing the isolated DNA in PCR analysis with specific primers we were able to amplify a product of the expected 469 bp size from the *MLL* locus on chromosome 11q23 (Figure 3B). There was no difference in PCR results between treatments.

Discussion

We describe here a modification of a rapid and inexpensive DNA isolation technique suitable for epidemiological studies. This method does not require time consuming preisolation of white blood cells. We simulated conditions of large scale epidemiological studies to investigate potential impact of the lag time between blood drawing and DNA isolation and the condition of storage of blood samples on the quantity and quality of isolated DNA.

Short-term storage is of interest for allowing batch processing to reduce effort and cost. The influence of short-term storage on yield and quality of DNA under 3 different conditions was assessed and compared to immediately isolated DNA. Our results indicate that short-term storage of blood cells at -80°C does not influence yield and purity of isolated DNA. Storage at 4°C, however, is less optimal with an approximately 20 % decrease in DNA yield. This has also been observed after 3 days of storage in a single experiment (4) and may be due to decreased viability of granulocytes (11). Addition and mixture with lysis buffer does not influence the amount and quality of isolated DNA and may be done depending on time requirements. The additional initial processing decreases the time needed for the actual isolation.

The amount of DNA we isolated from 4.5 ml whole blood was lower than originally described by McIndoe et al. (6) with this method. However, the amount of whole blood we used to isolate DNA was 10% less explaining some of the difference. Further, we did not vortex samples during the two hour incubation at 55°C. DNA yield can be increased up to two-fold by vortex mixing the samples every 30 minutes during

the incubation at 55°C as described in their protocol (data not shown). We attempted to keep the time of sample handling short. However, the additional effort introduced by this step is small and may, thus, be advisable to increase yield.

We performed Southern blot and PCR analysis after immediate DNA isolation and after storage under different conditions. There appeared to be no difference between treatments in suitability for genetic analyses using these techniques.

In summary, DNA isolated with this method is of good quality and well suitable for analyses in large epidemiological studies. The obtained amounts of DNA are currently sufficient to run at least 250 PCR analyses and are likely to be much higher in the future with improvements in PCR techniques. Processing of 50 - 70 samples requires a one-person workday. We recommend the use of this cost and time efficient technique to isolate DNA since large number of samples can be processed and short-term storage is without affect on DNA yield.

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Footnotes

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[4]The abbreviations used are: SST, serum separator tube; PCR, polymerase chain reaction; A_{260} , absorbance at 260 nm; A_{280}, absorbance at 280 nm.

Table 1. Mean (SD) DNA yield (μg) per subject by treatment

Subject	Treatment[a]				WBC[b] (10^3 cells/μl)
	A	B	C	D	
1	42.1 (12.2)	30.1[d] (6.6)	60.3 (12.4)	67.3 (10.5)	7.30
2	59.7 (34.6)	55.7 (7.7)	71.1 (9.9)	58.6 (45.0)	6.60
3[c]	27.0 (13.7)	33.0 (20.3)	31.1 (7.0)	31.9 (8.1)	4.30
4	67.9 (15.1)	57.7 (8.5)	57.4 (6.0)	57.3 (23.5)	6.70
5	56.9 (23.1)	35.9 (7.6)	34.5 (3.0)	55.0 (3.0)	5.20
6	59.3 (7.2)	46.9 (12.7)	45.7 (19.6)	45.4 (3.0)	5.80

[a]Values given are mean (SD) from triplicate samples.

A = immediate isolation of DNA; B = storage at 4°C for 7 days followed by isolation; C = storage at -80°C for 7 days followed by isolation; D = addition of 1 ml lysis/digestion buffer to the cells, mixture, and storage at -80°C for 7 days, followed by isolation.

[b]White blood cell count.

[c]Yield of subject 3 was significantly lower ($p < 0.05$) than in all other subjects in all treatments.

[d]Yield of subject 1 was significantly less ($p < 0.05$) in treatment B than in other treatments.

Table 2. Mean (SD) sample yield and purity of DNA by treatment[a]

Treatment	DNA (μg)[b]	A_{260}/A_{280}[b]
A	52.1 (14.9)	1.73 (.07)
B	43.2 (11.9)	1.74 (.12)
C	50.0 (15.6)	1.79 (.09)
D	52.6 (12.3)	1.74 (.09)
Mean	49.5 (13.4)	1.75 (.10)

[a]Values are based on the mean (SD) of the average of three 4.5 ml samples from 6 subjects in each treatment arm.

A = immediate isolation of DNA; B = storage at 4°C for 7 days followed by isolation; C = storage at -80°C for 7 days followed by isolation; D = addition of 1 ml lysis/digestion buffer to the cells, mixture, and storage at -80°C for 7 days, followed by isolation.

[b]No statistically significant differences in yield or purity were observed between treatments.

Figure 1. DNA processing in each of 6 subjects. 12 blood samples were drawn from each subject for DNA isolation.

Figure 2. Results of DNA electrophoresis are presented. Lane A to D on the left photograph show undigested DNA (2.5μg) from treatment A, B, C, and D, respectively. Molecular weight markers (kb) are depicted in the center lane. On the right hand side *BAM* HI endonuclease-digested samples from treatment A, B, C, and D, respectively, are depicted. Representative data of one subject (no. 1) are shown.

Figure 3. Panel A shows a Southern blot analysis of *BAM* HI digested DNA obtained through treatments A, B, C, and D. A fragment of the expected 8.3 kb size from the *MLL* locus on chromosome 11q23 was detectable using the 0.7 kb cDNA described in materials and methods. No apparent differences between treatments (A, B, C, and D) could be observed. Molecular weight markers (kb) are indicated. PCR with the described primers of DNA from treatments A, B, C, and D performed as described is depicted in Panel B. A control sample containing no DNA was loaded (control) and the λ *HIND*III (23 kb, 9.4kb, 6.6 kb, 2.3 kb, 2.0 kb, and 512 bp from top to bottom) molecular weight marker (marker) were run. A single band of 469 bp size was detected. No differences between treatments can be observed.

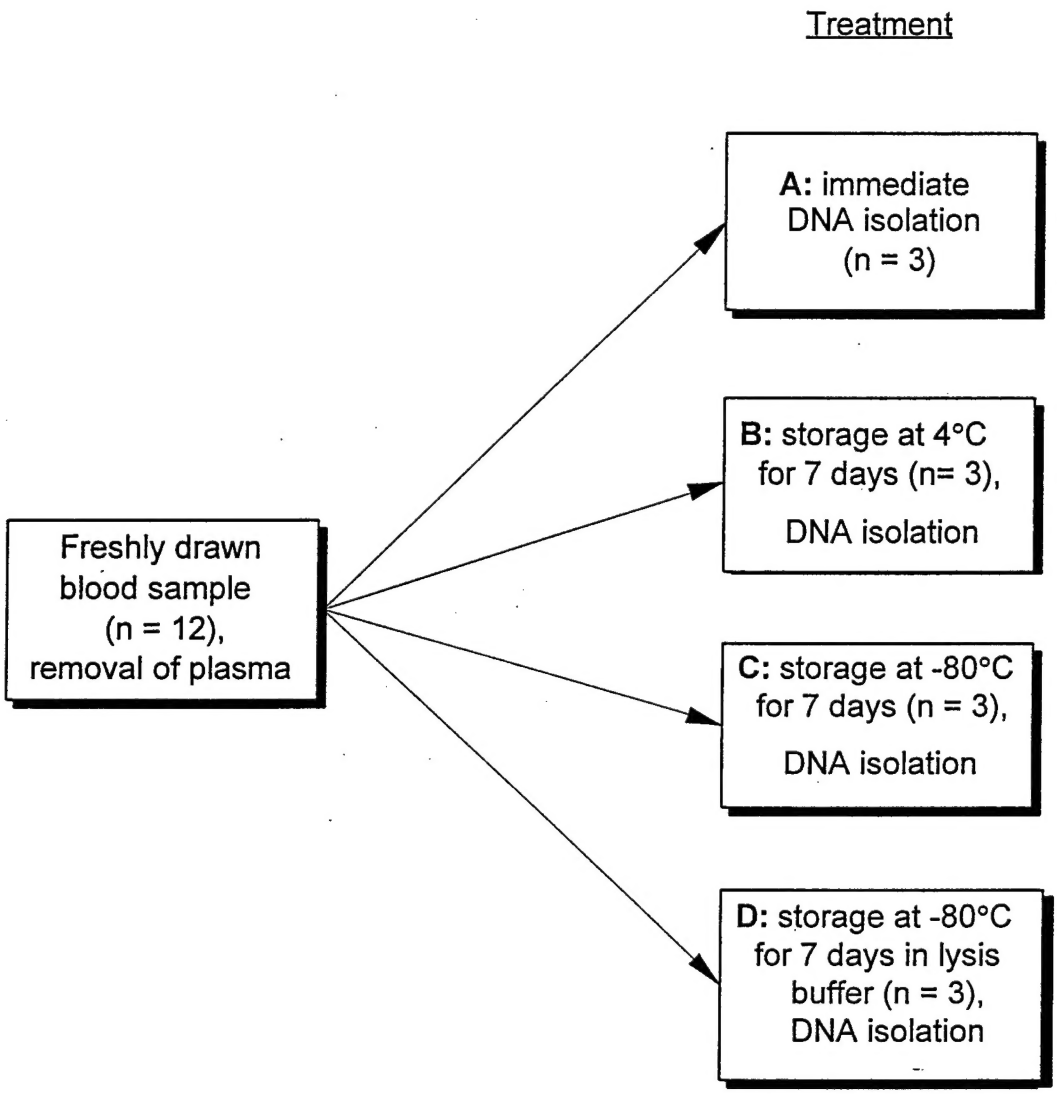
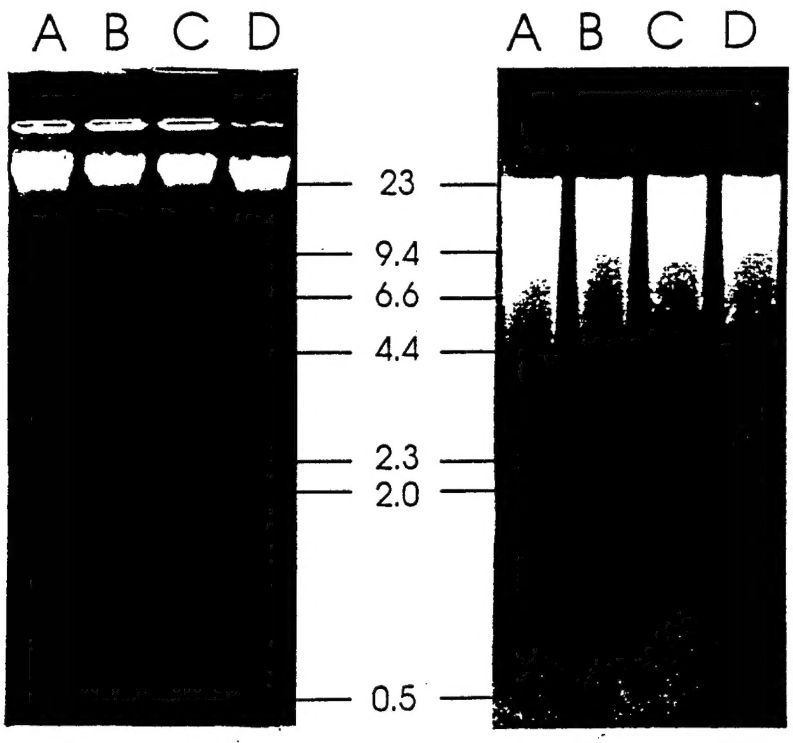

Treatment
A: immediate DNA isolation (n = 3)
B: storage at 4°C for 7 days (n= 3), DNA isolation
C: storage at -80°C for 7 days (n = 3), DNA isolation
D: storage at -80°C for 7 days in lysis buffer (n = 3), DNA isolation
Freshly drawn blood sample (n = 12), removal of plasma

Figure 1


A B C D
A B C D
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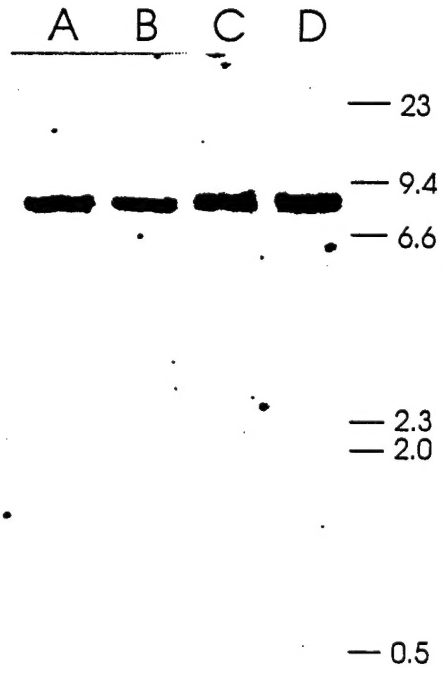
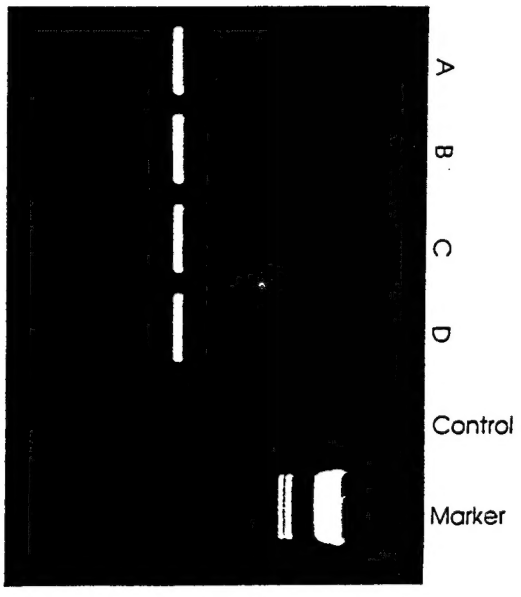
9.4
6.6
4.4
2.3
2.0
0.5


A B C D
23
9.4
6.6
2.3
2.0
0.5


A
B
C
D
Control
Marker